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Optimizing aerosolization of a high-dose L-arginine powder for pulmonary delivery

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ABSTRACT

In this study a carrier-free dry powder inhalation (DPI) containing L-arginine (ARG) was developed. As such, it is proposed that ARG could be used for adjunctive treatment of cystic fibrosis and/or tuberculosis. Various processing methods were used to manufacture high-dose formulation batches consisting various amounts of ARG and excipients. The formulations were evaluated using several analytical methods to assess suitability for further investigation. Several batches had enhanced in vitro aerolization properties. Significant future challenges include the highly hygroscopic nature of unformulated ARG powder and identifying the scale of dose of ARG required to achieve the response in lungs.

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Abbreviations: ACI, Andersen cascade impactor; API, active pharmaceutical ingredient; ARG, L-arginine; BM, ball milling; CI, Carr’s index; CF, cystic fibrosis; DoE, design of experiments; DPI, dry powder inhaler; DVS, dynamic vapour sorption; d10, particle diameter at which 10% of the particles have diameters that are greater or smaller than the d10 value; d50, particle diameter at which 50% of the particles have diameters that are greater or smaller than the d50 value; d90, particle diameter at which 90% of the particles have diameters that are greater or smaller than the d90 value; ED, emitted dose; FDA, U.S. Food and Drug Administration; FPD, fine particle dose; FPF, fine particle fraction; GRAS, Generally Recognized as Safe; HPMC, hydroxypropyl methylcellulose; HR, Hausner ratio; JM, jet milling; Leu, L-leucine; MF, mechanofusion; MS, magnesium stearate; M, moisture uptake; NO, nitric oxide; NOS, NO synthase; PCA, principal component analysis; PSD, particle size distribution; Q2, test set validation coefficient; RH, relative humidity; R2, correlation coefficient; SD, standard deviation; SEM, scanning electron microscopy; TB, tuberculosis; w/w, weight per weight; ρp, poured density; ρt, tapped density.

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1. **Introduction**

1.1. **Potential opportunities for inhaled arginine**

The pulmonary delivery of drugs represents a complex delivery challenge [1]; however, the advantages of this approach are well known and include rapid onset of action and delivery site targeting to minimize the dose [2]. For example an oral dose of bronchodilator may take 2–3 h to be effective, while an inhaled dose usually takes approximately 15–30 min [3]. More recently, new methods and materials for the formulation of dry powder particles suitable for highly-efficient high-dose pulmonary delivery have become available. High lung doses are traditionally delivered via a nebulizer; however, nebulizers are mostly restricted to use in hospital and ambulatory care settings due to their large size and expense. In addition, nebulized aerosols need to be delivered continuously over an extended period of time to provide a high dose. High dose delivery via a dry powder inhaler (DPI) would offer advantages in terms of size, ease of use, convenience and efficiency of delivery.

L-arginine (ARG) (Fig. 1) is one of the most common amino acids in nature. It is a semiessential or conditionally essential amino acid, depending on the developmental stage and health status of the individual [4]. ARG is used therapeutically in a range of human conditions [5] due to its conversion to nitric oxide (NO). NO mediates many human physiological processes including smooth muscle relaxation, bronchodilation [6], innate immune responses (direct mycobactericidal effect on bacterial cells) and cell-mediated immune effects (enhanced expression of the T cell receptor CD3ζ) [7]. This molecule has a crucial role specifically in human immune defense against *Mycobacterium tuberculosis* [7–9].

ARG shows promise as an inhaled therapeutic in cystic fibrosis (CF), a condition characterized by low pulmonary nitric oxide (NO) production [10–14]. CF is an autosomal recessive genetic disorder with high morbidity in the paediatric and young adult populations mostly due to chronic pulmonary infections. In several studies by Grasemann et al. [6,15] measurable pulmonary benefits in CF patients have been documented after targeted delivery of ARG to the lungs, whereby high-dose ARG was administered via nebulizer. The concentration of NO in exhaled air and forced expiratory volume in 1 second were both increased. ARG was well tolerated by paediatric patients [15].

Oral ARG has been trialed as an adjunctive therapy in people with pulmonary tuberculosis [16–19] in which, like CF, low pulmonary NO production has been documented [6]. Despite some initial promising findings [18,19], there were no benefits of ARG in the most recent trial using the highest ARG dose, and furthermore, oral ARG did not achieve any measurable increase in the concentration of NO in exhaled air [17]. It has been hypothesized that with higher doses, or targeted delivery to airways, ARG might be capable of measurably increasing pulmonary NO bioavailability, and thereby improving macrophage killing of tuberculosis bacilli. Therefore, inhaled ARG merits consideration as a means to generate elevated local pulmonary NO.

Proposed adjunctive therapies for CF or tuberculosis must be safe and effective, tolerated by adults and children, and also low-cost. Thus L-arginine is appealing as an investigational adjunctive agent in these conditions.

This study investigates possible formulation and processing approaches to enable the high dose delivery of ARG via a DPI.

1.2. **Creating high-dose inhaled formulations**

The principal criterion for a DPI is the ability of the system to efficiently and reproducibly deliver active pharmaceutical ingredient (API) to the target parts of the respiratory track to enable the required response. Particle aerodynamic size is the most important design variable for a DPI formulation [1]. To reach the peripheral airways, where drug is most efficiently absorbed, particles need to be below about 5 μm aerodynamic diameter [20]. According to Stokes Law, particles larger than 5 μm usually deposit in the oral cavity or pharynx by impaction and so do not reach the peripheral target. In addition, some deposition models suggest that particles smaller than 0.5 μm may not deposit efficiently, depending on the patient breathing pattern, and may be exhaled.

Most current DPI formulations consist of micronized drug blended with larger carrier particles, which enhance flow and aid in dispersion. However, inefficient drug/carryer separation is one of the main reasons for the relatively low deep lung deposition efficiency encountered with DPIs [21]. Also high dose delivery is a challenge in the performance of DPIs with carrier particles. Thus, in some cases it is preferred for no carrier particles to be present in the powder administered [22,23].

Product manufacture requires that powders have a sufficient flowability so that capsules for DPI devices can be easily and efficiently filled. Similarly, efficient aerosol delivery upon actuation of the DPI requires the formulation to have effective fluidization, such that it re-suspends and is entrained from the device and that effective de-aggregation occurs such that the API is detached from its carriers and/or itself once resuspended. However, micron-sized particles, particularly those resulting from high-energy operations such as jet milling, have high surface areas and tend to have high surface energies, which result in typically very poor flow and fluidization and a high disposition to form strong aggregates. Flow properties of material are influenced for example by particle size and size distribution, surface roughness and particle morphology, surface energy and also moisture/capillary effects [24]. Excipients can be used to reduce the cohesive nature of the API, and so enhance aerosolization efficiency by occupying the high-energy “sticky” surface sites of the drug particles. However, the range of effective potential excipients is limited for example to compounds that are endogenous, non-irritant and can easily be metabolized or cleared, and not requiring extensive toxicology evaluation. Excipients can be modified or combined within particle engineered structures with the API.

![Molecular structure of L-arginine.](image-url)
In addition, the relative humidity (RH) of air may have an impact on the flowability of small particles, especially with hygroscopic powders. The term ‘hygroscopicity’ describes the ability of a solid to take up and retain water [25]. Moisture uptake and loss due to changes in RH can result in local dissolution and recrystallization, leading to irreversible aggregation through solid bridge formation, which can adversely affect lung deposition [26–30]. Hygroscopicity can also alter the adhesive and cohesive properties, or, in more extreme situations, substantially increase particle size [31] or even cause dissolution. Excipients that modify the hygroscopic properties of a drug may need to be considered [1]. It has been indicated that the presence of hydrophobic additives may reduce the hygroscopic growth rate, the instantaneous particle size and by inference the deposition of aerosols in the lungs [32]. Several approaches have been proposed to produce micronized particles with hydrophobic surfaces that may reduce the problems present for hygroscopic drug particles [32–35].

In order to obtain a drug particle size in the respirable range, in most cases a size-reducing step is needed. There are several options for reducing the particle size, and it may be necessary to evaluate several methods to find the one most suited for the specific drug, dose and delivery environment.

Milling techniques are widely used methods in pharmaceutical industry. Respirable-sized particles are traditionally prepared by jet-milling techniques. Jet milling reduces particle size via high-velocity particle–particle collisions. Particles prepared by jet-milling can be highly electrostatically charged, comprise fractured crystals, and there is only a limited control over size, shape, and morphology of the produced particles [36].

Ball milling is an alternative micronization technique. Ball milling processes are less readily scalable and prone to media contamination, which is why they are mainly limited to the laboratory scale.

Mechanofusion, i.e. dry powder coating, is an alternative dry mechanical process step used to improve the flow of cohesive powders by modifying their inter-particle interactions. Mechanofusion process is described in detail by Zhou et al. [37]. The process imparts a considerable amount of thermomechanical energy that coasts the guest material onto the exposed surfaces of the host particles [38,39]. The mechanism of mechanofusion process is complex and is not well understood [40–42]. However, unlike general milling and co-milling processes, the energy input is more controlled and the process can be tuned to encourage coating but not size reduction [34]. Begat et al. [43] showed successful coating of micronized drug to achieve substantially enhanced drug aerosolization performance using additives such as magnesium stearate. Mechanical dry coating has also been successfully used for suppressing the hygroscopicity of materials [44,45].

Spray drying is growing in popularity as an alternative method for production of inhalable dry powder formulations due to its adaptability, cost-effectiveness and potential for complex particle engineering and scalability [46]. In spray-drying process the drug is dissolved in solvent, usually water, and sprayed as fine mist into a heated expansion chamber. The droplets dry, leaving behind tiny particles of drug that are collected. Spray dried particles can be formulated to contain various ingredients by adjusting the content and nature of the feed solution [47]. Thus, excipients can be incorporated to engineer the properties of the final powder. Co-spray drying a solution containing two drugs is a potential alternative to produce particles with uniform drug composition [48]. Thus spray drying is an attractive method for the manufacture of novel, sophisticated aerosol formulations.

The amino acid L-leucine is known to be an effective particle formation agent that substantially improves the processing yields of the spray dried formulations and forms high-rugosity particles [47]. It also has an ability to reduce cohesion and therefore enhance aerosolization efficiency and delivery [22,33,35,47–58]. Isomalt is a sugar alcohol which is used especially in hard candies but also in pharmaceutical tablets as an excipient due to its non-hygroscopic nature. Isomalt has Generally Recognized as Safe (GRAS) status but currently is not approved for pulmonary administration but only for oral tablets by the U.S. Food and Drug Administration (FDA). In this study isomalt was used to protect ARG from moisture effects in spray dried formulations.

For each particle size-reducing and surface-modifying technique it is important to consider a range of practical issues: for example, material effects, efficiency, reliability, scale, cost, supply chain and availability, etc. Spray drying offers greater flexibility and the possibility of morphology control but the yield can be low at small scale and the process is relatively expensive. Most commonly, milling remains the process of choice for micronizing drug because it is commonly available, easier to scale up, and is less expensive. However, it is well documented in the literature that milling can cause disruption of the crystal structure, leading to various degrees of disorder [59–62] and has the potential to influence electrostatic charge, flow, cohesiveness and polymorphic form including the amorphous form [63–65].

The aim of this study was to evaluate the effectiveness of these potential processing options in the context of developing a DPI for high-dose ARG delivery where requirements for the final DPI formulation focus are represented as follows:

1. Formulation should create a suitable aerosol to reach the lower parts of the respiratory tract efficiently
2. The amount of API has to be as high as possible, reflecting the anticipated large multi-milligram dose of API
3. The amount of excipients has to be as low as possible and powder bulk density is suitable to fit to the administration vessel (e.g. capsule for a DPI)
4. Materials should be well-tolerated in the lungs and approved or acceptable by suitable authorities
5. The materials and the manufacturing process have to be cost-effective and efficient at scale
6. The product should be physically and chemically stable in humid conditions to ensure suitability in all climactic conditions

2. Materials and methods

2.1. Materials

L-arginine (ARG, Sigma-Aldrich Chemicals, Castle Hill, Australia) was the active ingredient used in this study. Isomalt (food grade, Cake Deco, Melbourne, Australia), which is a disaccharide
consisting of glucose and mannitol, was used in spray drying as an excipient. Also L-leucine (Leu, Sigma-Aldrich Chemicals, Castle Hill, Australia) and magnesium stearate (MS, 2255, Mallinckrodt Specialty Chemicals, Dublin, Ireland) were used as excipients.

2.2. Methods

This formulation study involved several processing methods. The Design of Experiment (DoE) and nomenclature are represented in Fig. 2. Unprocessed ARG [ARG(raw)] was jet milled alone [JM(ARG)] and thereafter either ball milled or mechanofused with MS or Leu. ARG was also co-jet milled with Leu (ARG11) or MS (ARG12) and then ball milled or mechanofused. ARG was also spray dried with isomalt and Leu [SD(ARG)]. Furthermore SD(ARG) was mechanofused with MS (ARG10). In addition, ARG(raw) was studied as a reference.

2.2.1. Jet milling

Pure L-arginine [ARG(raw)] was jet milled to decrease the particle size. All of JM(ARG) used in this study was produced in one batch, avoiding potential batch differences. Thus, the batches from ARG1 to ARG9 were jet milled as a single primary source, prior to ball milling or mechanofusion. In addition, ARG11 and ARG12 were co-jet milled with excipients Leu (20% w/w) and MS (2% w/w), respectively.

The powders were jet milled using a Spiral Jet Mill 50 AS (Hosokawa Alpine AG, Augsburg, Germany). ARG and MS or ARG were manually mixed before the jet milling by tumbling for several minutes in a glass vessel. A grinding gas pressure of 7 bar and an injector gas pressure of 7.5 bar were used during the milling. The powder was fed to the jet mill using a vibrating feeder (Retsch Type DR100/75 Rinne rechts, Retsch GmbH, Haan, Germany).

2.2.2. Mechanofusion

Mechanofusion was carried out in AMS-Mini Mechanofusion system (Hosokawa Micron Corporation, Osaka, Japan) with Nobilta rotor blade (Hosokawa Micron Corporation, Osaka, Japan). The jet-milled ARG powder was mechanofused with excipients to manufacture batches ARG3 (2% MS), ARG4 (20% Leu) and ARG9 (20% MS). In addition batches ARG6 and ARG8 were mechanofused after co-jet-milling with excipients (excipients were added before jet-milling so no excipients were added for mechanofusion). Finally ARG10 was mechanofused after spray drying without added excipients in the mechanofusion phase (Fig. 2). The powders were premixed in the mechanofusion vessel for 5 min at 500 rpm. After premixing, the vessel was opened and the walls and lid were cleaned with a plastic brush. The speed of the blade was then increased from 0 to 4000 rpm for 1 min and was kept constant after the ramping for 10 min. Nitrogen flow rate was approximately 2.5 l/min and cold water was kept flowing through the incorporated water jacket to prevent the vessel temperature from exceeding 25 °C. The sample size was 10 g.

2.2.3. Ball milling

The jet-milled ARG powder was ball milled with excipients to manufacture batches ARG1 (20% Leu) and ARG2 (2% MS). In addition batches ARG5 and ARG7 were ball milled after co-jet-milling with excipients (excipients were added before jet-milling so no excipients were added for ball milling) (Fig. 2). Pulverisette 6 planetary mill (Fritsch Pulverisette, Idar Oberstein, Germany) was used for ball milling. The used bowl size was 250 ml and approximately 100 stainless steel balls (400 g) with diameter of 10 mm were used. Sample (10 g) was weighed on top of the balls in the bowl. Milling speed of 500 rpm was used for 3 min and after that the powder was separated from the balls with a sieve (size 300 μm).

2.2.4. Spray drying

An aqueous solution containing ARG, isomalt and Leu in w/w ratio 2:2:1 was dissolved in Milli-Q water so that a total 2.5% solids (w/w) concentration was achieved. This was mixed with a magnetic stirrer until the solution became clear. Spray-drying was conducted in a Buchi 190 spray dryer (Buchi, Labortechnik AG, Flawil, Switzerland) using the following standard operating conditions: air flow 800 l/h, solution flow 6.7 ml/min and aspirator pressure −84 mbar. These values lead to an outlet temperature of 70 (±5) °C. The powder was harvested in a humidity controlled space (RH 20 ± 2%, T = 23 °C). This spray dried batch was identified as SD(ARG). The processing yield was defined as the percentage of the mass of spray-dried powder recovered \( (M_{\text{recovered}}) \) compared to the mass of total solid loading \( (M_{\text{total}}) \) in the initial feed solution (Eq. 1):

\[
\text{Yield}\% = \frac{M_{\text{recovered}}}{M_{\text{total}}} \times 100
\]
2.2.5. Particle size
Particle size distributions (PSD) were measured by laser diffractometry using a Malvern Mastersizer system (Mastersizer 2000, Malvern Instruments Ltd., Malvern, UK) attached to a dry module (Scirocco, Malvern Instruments Ltd., Malvern, UK), and under optimized conditions, an inlet air pressure of 1.5 bar was used. Measurements were made in triplicate. The measurements were carried out at 50 ± 5% RH. The results are represented as d10, d50 and d90 values. These values are used as a quality control tool to provide a screening indication only to understand whether PSD is broadly within the respirable range.

2.2.6. Density and flowability
Poured density (ρp) and tapped density (ρt) were measured from the powders. The poured volume was measured by pouring approximately 10 g of the sample slowly into a 50 ml measuring cylinder (Glassco) via a funnel at a fixed height. ρt was calculated by dividing the mass of the sample with the poured volume. The cylinder was then attached to an automatic tapper (Autotap AT-2, Quantachrome Instruments, Boynton Beach, FL, USA) and tapped until the difference in the volume between these two taps was less than 2 ml typically 1250 taps (Ph. Eur.). The ρp was determined after tapping by dividing the mass of the sample with the tapped volume. Each sample was measured in triplicate. The Carr index (CI) [66] (Eq. 2) and the Hausner ratio (HR) [67] (Eq. 3) were calculated from ρt and ρp:

\[
CI = \frac{\rho_p - \rho_t}{\rho_t} ,
\]

\[
HR = \frac{\rho_t}{\rho_p} .
\]

2.2.7. Cohesion
The cohesion of each sample was characterized using the Freeman FT4 rheometer (Freeman Technology, Tewkesbury, UK) using a 1 ml shear cell. In the shear mode, a shear head was attached to the powder rheometer, and shear stress was measured with respect to the normal stress for a given consolidating stress. A fuller description of the principles of shear cell testing was described by Schwedes [68]. For this application a method described by Zhou et al. [37] was used. The cohesion value provides a measure of the cohesive inter-particle forces within the bed, and hence, a higher value corresponds to a more cohesive powder [68]. The FT4 measurements were carried out between 39% and 42% RH.

2.2.8. Morphology
Scanning electron microscopy (SEM) was used to investigate the particle size, shape and morphology of the samples. The powder was sprinkled on top of double-sided carbon tape and loose particles were removed by tapping the sample stub. Samples were prepared in 20 °C and 25 ± 2% RH. Samples were then coated with platinum in vacuum evaporation coater (Gun Quorum Q150TS, Quorum Technologies, Laughton, UK). The images were acquired using an acceleration voltage of 10 kV and magnification from 100× to 10,000× or 20,000× with FEI Quanta 250 FEG (FEI Inc., Hillsboro, OR, USA).

2.2.9. In vitro powder aerosolization
The in vitro powder aerosolization performance was determined using an abbreviated Andersen cascade impactor (ACI) system (Copley Scientific Limited, Nottingham, UK) configured as described and validated previously [47,69]. The Monodose inhaler (Miat S. p. A., Milan, Italy) was used as the aerosol dispersion device. It consisted of, from top to bottom, the throat piece, pre-separator (with 10 ml of water), stage 0, an impaction plate coated with a surfactant (Brij-35), stage F containing a filter paper, and the bottom plate. The cut-off aerodynamic diameter of powders deposited on the filter paper using this configuration at a flow rate of 90 l/min is approximately 4.7 μm according to the above literature. Approximately 20 mg of samples was weighed and filled into size 3 hydroxypropyl methylcellulose (HPMC) capsules (Capsugel, Peapack, NJ, USA). The capsule filling was performed in a humidity controlled environment (RH 20 ± 2%, T = 23 °C), and filled capsules were subsequently protected from moisture until firing.

The actuations were performed at 23 °C, 50 ± 5% RH. Each capsule was actuated from the inhaler over 6 s for each measurement. Measurements were made in triplicate. Fine particle dose (FPD) and fine particle fraction (FPF) were calculated as a percentage of the emitted dose (ED). FPD refers to the mass of drug in the potential deposition size range. In this study the effective cut-off diameter was 4.7 μm. FPF is the fraction of an aerosol that is in a size range (in this study 4.7 μm) with the potential to penetrate and deposit in the airways. ED and the amount of powder deposited on the filter paper were determined gravimetrically, an approach that is valid because no separate carrier particles were used in this formulation and stage loadings were sufficient to allow suitable measurement [47].

2.2.10. Dynamic vapour sorption and hygroscopicity
Dynamic vapour sorption (DVS) was used to evaluate the hygroscopicity of the powders. The measurements were performed using the DVS Intrinsic SMS (Surface Measurements Systems, London, UK) and were conducted under nitrogen gas at temperature of 25 °C. RH cycle 0 – 50 – 75 – 0% RH was employed. Time intervals were 70 – 140 – 110 – 100 min, respectively. Sample size varied from 16 mg to 20 mg.

Moisture uptake (M) was calculated from the weight (mw) recorded at time t = 320 min (at 75% RH) and the weight (m0) of the dried sample at time t = 70 min (at 0% RH) (Eq. 4):

\[
M_t = \frac{m_w - m_0}{m_0} \times 100
\]

2.2.11. Principal component analysis
Altogether 11 main results parameters were analysed using principal component analysis (PCA). These were moisture uptake (M), particle sizes (d10, d50, d90), densities (bulk and tapped), Hausner ratio (HR), Carr’s index (CI), cohesion, FPD and FPF. PCA categorized data so that similar samples are plotted at the same area in the score scatter plot to show the correlation between observations (in this case different batches). A loadings plot is used to show the relative influence of each variable (in this case different results parameters such as d10 or FPD) on the scores. The interpretation of the PCA at
3. Results and discussion

Fig. 2 provides a representation of all the test samples investigated and reported here.

3.1. Spray drying

The spray dried formulation for this study, SD(ARG), was chosen based on a series of unreported screening pre-tests. Prior to this study, 14 prototype batches were manufactured using different spray drying process parameters and excipients. The selected formulation, SD(ARG), was chosen because it had desirable particle size (according to the light scattering and ACI measurements), one of the highest yields and it was the least hygroscopic (according to the DVS measurements) compared to other batches in the pre-tests (results not shown). Leu was shown to improve the yields and made the powders generally easier to handle and aerolize. Pure ARG (with no excipients) was investigated as part of the pre-test regime but was found to be highly hygroscopic and delivered zero yield as it was unable to be recovered as dry particles from the collection cyclone.

The yield for SD(ARG) used in this study was 50.5% (w/w). The amount of aqueous solution for this batch of SD(ARG) was 400 g. When spray drying larger batch sizes (800 g), the yield was noticed to be slightly better (approximately 55%). The quality of powder, as measured by the powder characterization tests described here, appeared consistent regardless of the batch size.

Table 1 – Parameters related to particle size, density and flowability.

<table>
<thead>
<tr>
<th>Nomenclature</th>
<th>d10 (μm)</th>
<th>d50 (μm)</th>
<th>d90 (μm)</th>
<th>ρt (g/ml)</th>
<th>ρc (g/ml)</th>
<th>CI</th>
<th>HR</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARG1</td>
<td>2.4 (0.33)</td>
<td>14.0 (1.17)</td>
<td>56.3 (3.66)</td>
<td>0.29</td>
<td>0.54</td>
<td>47.2</td>
<td>1.89</td>
</tr>
<tr>
<td>ARG2</td>
<td>1.0 (0.01)</td>
<td>4.6 (0.04)</td>
<td>16.7 (0.52)</td>
<td>0.25</td>
<td>0.36</td>
<td>31.5</td>
<td>1.46</td>
</tr>
<tr>
<td>ARG3</td>
<td>0.9 (0.00)</td>
<td>2.5 (0.02)</td>
<td>6.3 (0.07)</td>
<td>0.25</td>
<td>0.40</td>
<td>38.0</td>
<td>1.61</td>
</tr>
<tr>
<td>ARG4</td>
<td>2.0 (0.02)</td>
<td>3.1 (0.20)</td>
<td>178.2 (6.79)</td>
<td>0.29</td>
<td>0.45</td>
<td>35.9</td>
<td>1.56</td>
</tr>
<tr>
<td>ARG5</td>
<td>2.4 (0.31)</td>
<td>15.5 (2.53)</td>
<td>102.4 (6.71)</td>
<td>0.34</td>
<td>0.58</td>
<td>41.9</td>
<td>1.72</td>
</tr>
<tr>
<td>ARG6</td>
<td>1.0 (0.05)</td>
<td>2.4 (0.05)</td>
<td>5.9 (0.29)</td>
<td>0.26</td>
<td>0.45</td>
<td>41.1</td>
<td>1.70</td>
</tr>
<tr>
<td>ARG7</td>
<td>1.0 (0.02)</td>
<td>4.1 (0.23)</td>
<td>15.9 (0.96)</td>
<td>0.21</td>
<td>0.43</td>
<td>51.6</td>
<td>2.06</td>
</tr>
<tr>
<td>ARG8</td>
<td>0.9 (0.03)</td>
<td>2.1 (0.04)</td>
<td>4.6 (0.31)</td>
<td>0.22</td>
<td>0.33</td>
<td>34.1</td>
<td>1.52</td>
</tr>
<tr>
<td>ARG9</td>
<td>1.1 (0.01)</td>
<td>3.9 (0.05)</td>
<td>11.4 (0.12)</td>
<td>0.28</td>
<td>0.50</td>
<td>43.4</td>
<td>1.77</td>
</tr>
<tr>
<td>ARG10</td>
<td>1.3 (0.02)</td>
<td>2.9 (0.02)</td>
<td>6.7 (0.08)</td>
<td>0.28</td>
<td>0.45</td>
<td>39.0</td>
<td>1.64</td>
</tr>
<tr>
<td>ARG11</td>
<td>1.0 (0.01)</td>
<td>2.4 (0.03)</td>
<td>5.4 (0.10)</td>
<td>0.20</td>
<td>0.27</td>
<td>25.3</td>
<td>1.34</td>
</tr>
<tr>
<td>ARG12</td>
<td>1.0 (0.03)</td>
<td>2.7 (0.13)</td>
<td>6.5 (0.31)</td>
<td>0.17</td>
<td>0.23</td>
<td>25.0</td>
<td>1.33</td>
</tr>
<tr>
<td>JM(ARG)</td>
<td>1.0 (0.06)</td>
<td>2.8 (0.19)</td>
<td>14.9 (15.64)</td>
<td>0.16</td>
<td>0.21</td>
<td>21.7</td>
<td>1.28</td>
</tr>
<tr>
<td>SD(ARG)</td>
<td>1.2 (0.01)</td>
<td>2.3 (0.01)</td>
<td>4.3 (0.03)</td>
<td>0.11</td>
<td>0.16</td>
<td>32.0</td>
<td>1.47</td>
</tr>
<tr>
<td>ARG(raw)</td>
<td>12.4 (1.10)</td>
<td>72.7 (0.85)</td>
<td>232.0 (0.10)</td>
<td>0.63</td>
<td>0.82</td>
<td>24.0</td>
<td>1.32</td>
</tr>
</tbody>
</table>

d10 = particle diameter at which 10% of the particles have diameters that are greater or smaller than the d10 value, represented as mean (μm), SD in parentheses (n = 3); d50 = particle diameter at which 50% of the particles have diameters that are greater or smaller than the d50 value, represented as mean (μm), SD in parentheses (n = 3); d90 = particle diameter at which 90% of the particles have diameters that are smaller than the d90 value, represented as mean (μm), SD in parentheses (n = 3); ρt = poured density (g/ml) (n = 1); ρc = tapped density (g/ml) (n = 1); CI = Carr index (n = 1); HR = Hausner ratio (n = 1).

3.2. Particle size and shape

Particle size (d10, d50 and d90) of the samples is represented in Table 1. ARG(raw) had a large particle size (d50 = 72.7 μm, n = 3) compared to the processed batches. SEM images seen in Fig. 3 also provide a useful assessment of particle size.

Jet milling decreased the particle size of ARG substantially. Ball milling did not seem to further decrease the particle size beyond that of the jet-milled samples but it changed the shape of the particle according to SEM images (Fig. 4). Mechanofusion did not have an effect on the particle size or shape of the powders.

The particle sizes of the processed batches as indicated by d50 were all generally satisfactory for pulmonary delivery, with d50 being less than 5 μm, except for ARG1 and ARG5 which had d50 values greater than 10 μm. d values are provided here as a quality control tool to provide a screening indication only to understand whether PSD is broadly within the respirable range. It is worth noting that d values become inappropriate as an indicator for the respirable range only when very low density or very high density particles are used, or where shape factors are extreme deviations from sphericity. None of these are particularly relevant to any of the composite Leu particles here, so d values were argued to be acceptable just as screening indicator for this work.

In addition, ARG1, ARG4 and ARG5 had large d90 values in excess of 100 μm, and ARG2 and ARG7 had d90 values above 15 μm. SEM images (with magnification of 500× to 1000×) revealed that there were large particles evident in the samples ARG1, ARG2, ARG5 and ARG7 (Fig. 5) and it appeared possible that these ball milling samples exhibited hard agglomerates created during processing. Aggregates were occasionally also present in other samples such as ARG4 due to hygroscopicity of the materials.

In general, from SEM images the jet-milled and mechano-fused samples appeared needle shaped, with individual needles...
approximately 1 × 3 to 10 μm. Ball-milled samples appeared more irregularly shaped, with lower aspect ratio than the needles. Thus, the mechanofusion process appeared less shape modifying than the ball milling process. Spray dried samples (SD(ARG) and ARG10) appeared as spherically aggregated particles with individual particles approximately 2 μm diameter.

3.3. Density and flowability

Bulk and tapped density and flowability of the batches are represented in Table 1. Unmodified ARG was the most dense, while SD(ARG) had the lowest density. When SD(ARG) was mechanofused with MS (ARG10), the density increased substantially. Jet milled samples started with low density, and after further processing (BM or MF), the densities also increased.

HR and CI are both proposed as measures of the flow properties of powders. HR of < 1.25 should indicate a powder that is free flowing, whereas > 1.25 indicates poor flow ability. Similarly, the smaller CI, the better the flow properties. It has previously been reported that the apparent relationship between particle size and flow behavior for micronized highly cohesive powders is generally not observed in CI and HR values [37]. The current work similarly suggests no consistent pattern to support this, and it appears that cohesivity is so high that tapping is not sufficient to measurably alter the density and provide a good indicator of flow. Consequently, cohesion was also measured using the Freeman FT4 rheometer shear cell.

3.4. Cohesion

Unmodified ARG, due to its larger particle size, had the lowest cohesion (Table 2). It was noted that the cohesion values for powders processed with MS were relatively lower than cohesion values for corresponding formulations with Leu. Thus MS was deemed to reduce the interaction between ARG particles better than Leu, potentially by its reported capability to better cover the particle surface with a hydrophobic layer [71]. Lubrication of dry coating of particle surface has been demonstrated to modify the surface energy of pharmaceutical powders [37,72,73]. Jet-milled batches had higher cohesion than mechanofused and ball-milled batches. It was observed that further processing with lubricant decreased the cohesion (excluding ARG8), this effect being most pronounced with ARG9 containing 20% of MS.

3.5. In vitro powder aerolization

In vitro powder aerolization results are represented in Table 2. The emitted powder doses (EP) for all formulations were above 60%, and mostly between 75 and 90%. Thus, powders were generally well fluidized and emitted from the capsule and the device.

In each case, the theoretical amount of excipients was taken into account when calculating FPD and FPF. For instance SD(ARG) included only 40% ARG, so the actual amount of ARG was only less than 7 mg (of approximately 20 mg weighed into the capsule). However, its EP was almost 95% and total powder FPF was more than 90%, indicating excellent aerosolization behavior. Batches ARG3, ARG6, ARG8 and ARG11 had the highest FPD values providing 9–10 mg of ARG on the filter paper ACI stage. In general, mechanofused and co-jet milled samples gave much higher FPF and FPD values than ball-milled samples. There were no consistent differences between the used excipient (MS or Leu) and FPD or FPF, with both providing improved aerosolization. The addition of MS or Leu in jet milling gave a substantial improvement compared to pure JM(ARG).
Fig. 4 – SEM images of the samples. (A) ARG1, (B) ARG2, (C) ARG3, (D) ARG4, (E) ARG5, (F) ARG6, (G) ARG7, (H) ARG8, (I) ARG9, (J) ARG10, (K) SD(ARG), (L) JM(ARG), (M) ARG11, and (N) ARG12. Magnification 5000×, scale bar in the images.
According to the FPD results, the best performing batches (FPD > 9 mg) were ARG3, ARG6, ARG8 (all mechanofused) and ARG11 (jet milled with Leu). These were substantially better than most other formulations, which had FPD generally below 4 mg.

Standard deviation (SD) for FPD values indicated good measurements repeatability ($n = 3$). Only ARG4 had an SD over 1 and this may be explained with high d90 value referring to the observed large particles in the sample.

As expected when particle size (d50) decreased, generally FPF and FPD increased and vice versa. ARG1 and ARG5 had significantly larger particle sizes (d50) than the other batches and consequently demonstrated low FPF. ARG10 was an exception as the particle size of this formulation was small but FPF is poor since the batch showed relatively high hygroscopicity ($M_t = 22.77\%$). It can be also stated that there was no observable relationship between density-derived flowability (CI and HR) and FPF.

In this study, the jet-milled needle-shaped particles were observed to achieve good dispersion during the actuation. Louey et al. [74] studied the effect of jet milling and spray drying of mannitol particles for pulmonary delivery and concluded that the aerosol dispersion of angular jet-milled particles was not as efficient as spherical spray-dried particles, with spray-dried particles producing higher values of FPF. In this study, the spray dried particles give excellent aerosolization but are limited in FPF due to the amount of excipient required.

### 3.6. Dynamic vapour sorption and hygroscopicity

DVS results are expressed as the weight increase from 0% RH to 75% RH ($M_t$) and values are represented in Table 2. The most hygroscopic samples were both spray dried samples: ARG10 ($M_t = 22.77\%$) and SD(ARG) ($M_t = 24.38\%$). Any moisture protection by mechanofusion with MS was not detectable based on the conditions used in this test. The least hygroscopic samples were ARG4, ARG2, ARG8, ARG11, ARG7 and ARG9 ($M_t = 15.57–16.49\%$). For the rest of the samples, $M_t$ was between 19.14 and 20.01%.

**Table 2 – Parameters related to in vitro aerolization, cohesion and moisture uptake.**

<table>
<thead>
<tr>
<th>Nomenclature</th>
<th>Process</th>
<th>Excipient(s)</th>
<th>FPF (%)</th>
<th>FPD (%)</th>
<th>ED (%)</th>
<th>EP (%)</th>
<th>Cohesion (kPa)</th>
<th>$M_t$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARG1</td>
<td>JM→BM</td>
<td>MS</td>
<td>8.47 (0.86)</td>
<td>1.51 (0.01)</td>
<td>17.90 (1.73)</td>
<td>87.14 (6.86)</td>
<td>1.45</td>
<td>19.73</td>
</tr>
<tr>
<td>ARG2</td>
<td>JM→BM</td>
<td>Leu</td>
<td>26.77 (0.48)</td>
<td>3.94 (0.29)</td>
<td>14.70 (0.97)</td>
<td>90.86 (1.84)</td>
<td>1.54</td>
<td>16.26</td>
</tr>
<tr>
<td>ARG3</td>
<td>JM→MF</td>
<td>MS</td>
<td>69.51 (1.57)</td>
<td>10.54 (0.74)</td>
<td>15.16 (0.89)</td>
<td>73.56 (1.49)</td>
<td>1.78</td>
<td>19.76</td>
</tr>
<tr>
<td>ARG4</td>
<td>JM→MF</td>
<td>Leu</td>
<td>57.49 (10.28)</td>
<td>8.13 (1.56)</td>
<td>14.13 (0.76)</td>
<td>86.29 (1.67)</td>
<td>3.07</td>
<td>15.57</td>
</tr>
<tr>
<td>ARG5</td>
<td>JM→BM</td>
<td>MS</td>
<td>4.00 (0.68)</td>
<td>0.78 (0.16)</td>
<td>19.35 (0.77)</td>
<td>100.17 (0.28)</td>
<td>1.24</td>
<td>19.65</td>
</tr>
<tr>
<td>ARG6</td>
<td>JM→MF</td>
<td>MS</td>
<td>63.40 (5.53)</td>
<td>9.88 (0.27)</td>
<td>15.64 (0.90)</td>
<td>77.83 (1.14)</td>
<td>1.28</td>
<td>19.78</td>
</tr>
<tr>
<td>ARG7</td>
<td>JM→BM</td>
<td>Leu</td>
<td>26.70 (0.61)</td>
<td>4.02 (0.16)</td>
<td>15.08 (0.91)</td>
<td>91.82 (1.97)</td>
<td>2.38</td>
<td>16.38</td>
</tr>
<tr>
<td>ARG8</td>
<td>JM→MF</td>
<td>Leu</td>
<td>67.02 (3.09)</td>
<td>9.98 (0.14)</td>
<td>14.91 (0.65)</td>
<td>84.40 (3.60)</td>
<td>3.88</td>
<td>16.31</td>
</tr>
<tr>
<td>ARG9</td>
<td>JM→MF</td>
<td>MS20%</td>
<td>30.13 (0.62)</td>
<td>3.75 (0.18)</td>
<td>12.46 (0.76)</td>
<td>76.41 (2.63)</td>
<td>1.08</td>
<td>16.49</td>
</tr>
<tr>
<td>ARG10</td>
<td>SD→MF</td>
<td>Leu, IM→MS</td>
<td>18.24 (1.63)</td>
<td>1.29 (0.08)</td>
<td>7.10 (0.21)</td>
<td>91.59 (1.74)</td>
<td>10.88</td>
<td>22.77</td>
</tr>
<tr>
<td>ARG11</td>
<td>JM</td>
<td>Leu</td>
<td>62.15 (0.60)</td>
<td>9.32 (0.40)</td>
<td>15.01 (0.78)</td>
<td>85.24 (3.02)</td>
<td>3.13</td>
<td>16.35</td>
</tr>
<tr>
<td>ARG12</td>
<td>JM</td>
<td>MS</td>
<td>49.37 (1.92)</td>
<td>7.77 (0.54)</td>
<td>15.76 (1.40)</td>
<td>79.30 (1.98)</td>
<td>2.54</td>
<td>19.14</td>
</tr>
<tr>
<td>JM(ARG)</td>
<td>JM</td>
<td>−</td>
<td>48.88 (3.85)</td>
<td>5.86 (0.68)</td>
<td>11.98 (0.71)</td>
<td>60.71 (4.31)</td>
<td>3.41</td>
<td>20.01</td>
</tr>
<tr>
<td>SD(ARG)</td>
<td>SD</td>
<td>Leu, IM</td>
<td>90.21 (4.89)</td>
<td>6.88 (0.14)</td>
<td>7.64 (0.38)</td>
<td>94.71 (2.44)</td>
<td>3.70</td>
<td>24.38</td>
</tr>
<tr>
<td>ARG(raw)</td>
<td>−</td>
<td>−</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>0.72</td>
<td>19.99</td>
</tr>
</tbody>
</table>

MS = magnesium stearate; Leu = leucine; IM = isomalt; JM = jet milling; BM = ball milling; MF = mechanofusion; EP = emitted powder (%), represented as mean, SD in parentheses ($n = 3$); FPD = fine particle dose (mg), represented as mean, SD in parentheses ($n = 3$); FPF = fine particle fraction (%), represented as mean, SD in parentheses ($n = 3$); ED = emitted dose (mg), represented as mean, SD in parentheses ($n = 3$); Cohesion = cohesion measured using FT4 (kPa) ($n = 1$); $M_t$ = moisture uptake (%) ($n = 1$); n/a = not available.
Based on the standards described by Newman et al. [75], all of the batches were deemed to be very hygroscopic (>15%, w/w, of moisture uptake) (with no batches being classified as extremely hygroscopic (>25%, w/w, moisture uptake).

Most of the batches that contained Leu had less moisture absorbed (lower Mt values) than batches with MS. However, ARG9, which included 20% MS, had lower moisture uptake than the other batches with MS, including ARG3 which was processed exactly the same as ARG9, with the only difference being the amount of MS. Thus, a higher amount of MS used seemed to protect ARG better from moisture.

Interestingly, there was no observed correlation between Mt and FPD or FPF. Mt did not correlate with cohesion either, even though it is known that moisture can increase cohesion of fine particles. This indicated that moisture uptake was not of a nature to affect the in vitro powder aerosolization performance. This could be due to moisture uptake being internal rather than collecting at the surface.

3.7. Principal component analysis

PCA was established based on the parameters described in Section 2.2.11. The $R^2$ (correlation coefficient) value with two principal components in the model was 67.3% and $Q^2$ value (test set validation coefficient) was 54.5%, indicating a good model.

Formulation batches with similar behavior according to result parameters (such as $d_{10}$ or FPD) appear in the same area in the score scatter plot (Fig. 6A). The result parameters that account for the distribution on the score plot are plotted in the loading plot (Fig. 6B and 6C). For instance batches ARG1 and ARG5 are plotted in the right side of the score plot due to their similar $d_{50}$ values. Thus the loading plot reveals covariance among variables and can be used to interpret patterns observed in the score plot.

The results of this PCA suggest that, if FPF and FPD were emphasized as the most important parameters for the formulation, batch ARG8 would be the preferred candidate, with batches ARG3, ARG6 and ARG11 as close alternative candidates.

The optimized co-spray dried batch produced particles with a suitable particle size distribution and excellent in vitro aerosolization performance. However, due to the hygroscopicity of ARG, the composition was diluted substantially and so achieving high dose delivery of ARG using a spray drying approach may be challenging. In this study excipients (40% (w/w) isomalt and 20% (w/w) Leu) were used for spray drying when only 2% (w/w) of MS or 20% (w/w) of Leu was needed for jet-milling, mechanofusion and ball milling. Thus the amount of ARG in the final formulation is substantially different. Furthermore, it will need to be established from a toxicology and hence regulatory basis which spray drying excipients (such as isomalt) are suitable for (pulmonary) delivery.

Jet milling produced micronized ARG particles but co-jet milling with suitable additives was shown to create particles that were more suitable for high-dose efficient aerosolization. In this case, a much lower excipient load more than compensated for slightly lower aerosolization efficiency relative to optimized spray-drying. As a subsequent step, mechanofusion formulations appeared to provide ARG with more protection from moisture, and this may be due to a more coherent particle coating than with co-jet milling. Ball milling was studied as a potential alternative, but substantially reduced the in vitro aerosolization performance, which appeared to result from caking of the powders as seen in SEM images (Fig. 4).

In this study, the aerosolization parameters were calculated from gravimetric data – an approach that was valid because no separate carrier particles were incorporated in the formulation. Nevertheless, the formulations used in this study contained different amounts excipients which might lead to different amounts/ratios depositing to different parts of the lungs if the excipients were not evenly distributed in the formulation. With respect to spray drying, during spray drying of a solution the formation of particles from droplets of a homogeneous solution very rarely can provide any credible
mechanism for the separation of those homogeneously distributed components, and so for spray drying there would be no substantial partitioning expected. Mechanofusion has been previously reported for similar studies [37] and is regarded to create host–guest structures with the excipient additives, such as MS or Leu used here, forming robust complete or partial coating layers, and so partitioning is not expected. Both MS and Leu are soft lamellar waxy solids, which readily adhere to host surfaces. Furthermore, with MS, formulations comprised only 2% (w/w) of excipient, and so any segregation/partitioning was most unlikely to have measurable impact on the data outside the expected noise in the data. For the ball milled and co-jet milled samples, such host–guest structures are also expected, but there is less experience in the literature to support this, and so for the cases with Leu where 20% is added, there is a degree of greater uncertainty here, but given that the composition was 20% (w/w) excipient, again any absolute error here was likely small, and this would have affected samples ARG1, ARG4, ARG7-ARG9 and ARG11. On reviewing the FPD data, ARG8 and ARG11 were the most prone to this issue, although ARG8 was mechanofuscated which would mean that it was arguably less prone to partition.

The prevalence of TB is highest in regions classified as climatic zones III, IV, and IVb by the International Commission for Harmonization of Laboratory Data [76]. This means that a formulation for this indication would need to function effectively during administration at elevated humidity conditions. Protection from humidity during storage can be achieved by foil wrap packaging, although extra cost for packaging would incur. Further studies should test whether the short term high-humidity exposure of the powder during actuation would significantly compromise aerosolization.

An oral dose for ARG in the study by Ralph et al. [16] was as high as 6 g. Amounts this high are most likely impractical by inhalation. Traditionally, in order for a drug to be considered for inhalation therapy, it needs to be therapeutically effective in the low microgram or the milligram range for a single dose [77]. However, studies have shown from other areas that inhaled doses may be an order of magnitude reduced relative to oral, and this may be reduced further for a highly efficient inhaled formulation [3]. With the development of new inhaler technologies, this quantity may increase substantially [78] and limitation may be more related to patient tolerance of the inhaled mass. Surprisingly large amounts (total 112 mg) of spray-dried tobramycin powder were administered to CF patients using Novartis T-326 Inhaler. This formulation has been shown to be well tolerated in patients [79].

In our study, we have shown that more expensive particle engineering technologies such as spray drying or emulsion/precipitation may not be necessary for producing a readily aerosolizable ARG formulation, capable of aerosolizing milligram doses at high respirable fraction efficiencies. Increased powder bulk densities, such as achieved by mechanical powder coating, may also aid in producing suitable metred unit doses.

In addition to total dose, drug targeting should also be considered. For TB, the mycobacteria exist in macrophages in the lungs. The question rises, should ARG be targeted in a format, perhaps employing extended release, to access the macrophages to gain better response?

4. Conclusions

Suitable in vitro aerosolization properties were achieved with several prototype DPI formulations of ARG. Co-Jet milling or jet milling combined with mechanofusion to produce coated ARG particles was the most promising manufacturing method. Spray dried batches gave excellent aerosolization but required dilution with excipient such that the ARG dose was lower. In addition, powders manufactured using spray drying were sensitive to moisture.

The excipients magnesium stearate and L-leucine were suitable coating additives for the formulation. However, the hygroscopicity of ARG might still be a challenge even when using these protective excipients. It also remains to be seen what will be the required dose for ARG and if this high demand be fulfilled when utilizing a conventional DPI.

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