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Abstract: The high hygroscopicity of gentamicin (G) as raw material hampers the production of respirable particles during aerosol generation and prevents its direct use as powder for inhalation in patients suffering from cystic fibrosis (CF). Therefore, this research aimed to design a new dry powder formulation of G studying dispersibility properties of an aminoacid, L-leucine (leu), and appropriate process conditions. Spray-dried powders were characterized as to water uptake, particle size distribution, morphology and stability, in correlation with process parameters. Aerodynamic properties were analyzed both by Single Stage Glass Impinger and Andersen Cascade Impactor. Moreover, the potential citotoxicity on bronchial epithelial cells bearing a CFTR F508/ F508 mutant genotype (CuFi1) were tested. Results indicated that leu may improve the aerosol performance of G-dried powders. The maximum fine particle fraction (FPF) of about 58.3 % was obtained when water/isopropyl alcohol 7:3 system and 15-20% w/w of leu were used, compared to a FPF value of 13.4 % for neat G-dried powders. The enhancement of aerosol efficiency was credited both to the improvement of the powder flowability, caused by the dispersibility enhancer (aminoacid), and to the modification of the particle surface due to the influence of the organic co-solvent on drying process. No significant degradation of the dry powder was observed up to 6 months of storage. Moreover, particle engineering did not affect either the cell viability or cell proliferation of CuFi1 over a 24 h period.

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Dear Editor,
We are pleased to submit you the manuscript entitled

DRY POWDER INHALERS OF GENTAMICIN AND LEUCINE: FORMULATION PARAMETERS, AEROSOL PERFORMANCE AND IN VITRO TOXICITY ON CUFI1 CELLS.

by

R.P. Aquino[a], L. Prota[a], G. Auriemma[a], A. Santoro[a], T. Mencherini[a], G. Colombo[b], P. Russo[a*]

We would be glad if you will consider it for the publication on International Journal of Pharmaceutics.
Research addresses the preparation, characterization and optimization of dry powders of Gentamicin, as a valid alternative to antibiotics already used in the therapy against Pseudomonas aeruginosa, an opportunistic pathogen showing frequent drug resistance phenomena. Gentamicin, an aminoglycosidic antibiotic, is widely used against Pa infections even if it shows low bioavailability when administered *per os* and toxicity problems if administered intravenously in quantities able to determine in the lung the Minimum Inhibiting Concentration.

Therefore, this research aimed to design a new dry powder formulation of G, studying dispersibility properties of an aminoacid, leucine, and appropriate spray-drying conditions. Moreover, the effect of the produced powders on cell viability and cell proliferation of bronchial epithelial cells bearing a CFTR F508/ F508 mutant genotype (CuFi1) was evaluated by MTT and ELISA assays, respectively.

All the Authors declare there are no conflict of interest to disclose.

Yours sincerely,
Paola Russo

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The authors appreciated the reviewer comments that allow the manuscript to be substantially improved. The specific comments raised by the reviewer have been punctually addressed and the author comments are hereunder reported.

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1. The operating mechanisms and basic functionality of the device chosen for the in vitro deposition studies (TURBOSPIN) have been reported in the revised manuscript.

2. When possible, 'micronized powders' has been replaced by 'spray-dried powders'.

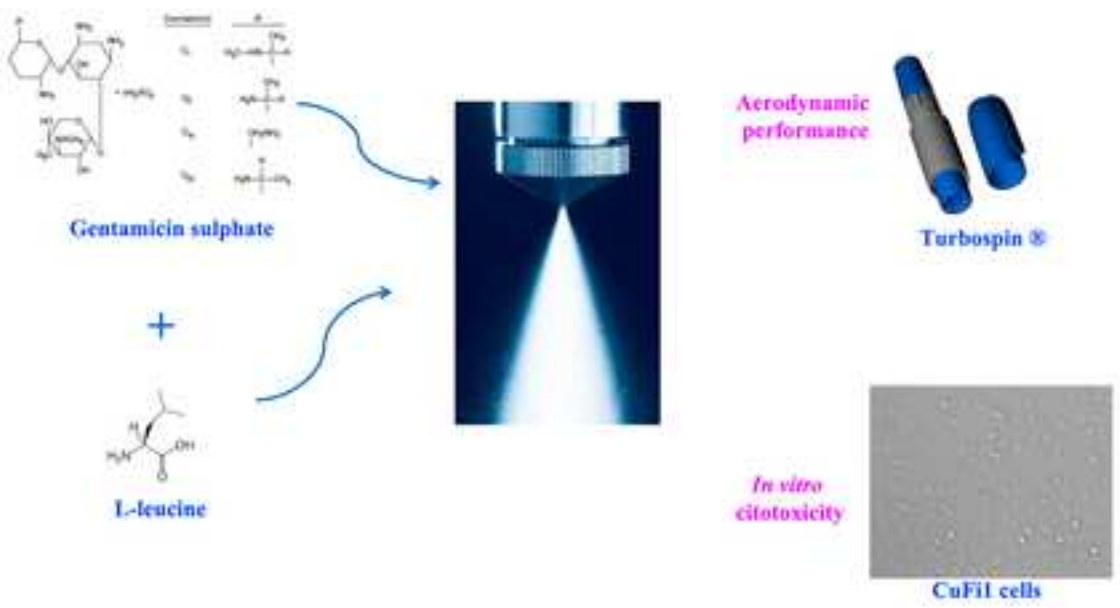
3. The Table 2, showing how solution compositions affected the physical characteristics of spray dried particles, has been included.

4. The correlation between physical characteristics and aerodynamic properties of spray-dried powder has been discussed in the results session.

5. EDs, FPFs and FPDs values of various spray dried powders before and after 6 month storage at the CRT conditions have been added in the Table 4.

6. Figures 1 and 4 have been deleted.

*Graphical Abstract



DRY POWDER INHALERS OF GENTAMICIN AND LEUCINE: FORMULATION PARAMETERS, AEROSOL PERFORMANCE AND IN VITRO TOXICITY ON CUFI1 CELLS.

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Keywords: Cystic Fibrosis; Gentamicin sulphate; L-leucine; spray drying; dry powder inhaler; CF airway epithelial cells.

Abbreviations: CF, cystic fibrosis; ACI, Andersen cascade impactor; CFTR, cystic fibrosis transmembrane conductance regulator; DPI, dry powder inhaler; ED, emitted dose; FPD, fine particle dose; FPF, fine particle fraction; G, Gentamicin sulphate; IPA, isopropyl alcohol; leu, L-leucine; MMAD, mass median aerodynamic diameter; SEM, scanning electron microscopy; SSGI, single stage glass impinger.

ABSTRACT

The high hygroscopicity of gentamicin (G) as raw material hampers the production of respirable particles during aerosol generation and prevents its direct use as powder for inhalation in patients suffering from cystic fibrosis (CF). Therefore, this research aimed to design a new dry powder formulation of G studying dispersibility properties of an aminoacid, L-leucine (leu), and appropriate process conditions. Spray-dried powders were characterized as to water uptake, particle size distribution, morphology and stability, in correlation with process parameters. Aerodynamic properties were analyzed both by Single Stage Glass Impinger and Andersen Cascade Impactor. Moreover, the potential citotoxicity on bronchial epithelial cells bearing a CFTR F508/ F508 mutant genotype (CuFi1) were tested. Results indicated that leu may improve the aerosol performance of G-dried powders. The maximum fine particle fraction (FPF) of about 58.3 % was obtained when water/isopropyl alcohol 7:3 system and 15-20% w/w of leu were used, compared to a FPF value of 13.4 % for neat G-dried powders. The enhancement of aerosol efficiency was credited both to the improvement of the powder flowability, caused by the dispersibility enhancer (aminoacid), and to the modification of the particle surface due to the influence of the organic co-solvent on drying process. No significant degradation of the dry powder was observed up to 6 months of storage. Moreover, particle engineering did not affect either the cell viability or cell proliferation of CuFi1 over a 24 h period.

1. Introduction

Pulmonary infections are the major cause of morbidity and mortality in cystic fibrosis (CF), with *Pseudomonas aeruginosa* (*Pa*) acting as the principal pathogen. The viscous mucus lining the lung of CF patients impairs the mucociliary function, causing recurrent and chronic respiratory infections caused mainly by *Pa* but also by *Haemophilus influenzae*, *Bulkolderia cepacia*

(Mukhopadhyay et al., 1996; Ramsey et al., 1999). Antibiotic treatment is an accepted standard in CF cure aiming at reducing decline in lung function and number of hospitalizations (Prayle and Smyth, 2010). Aminoglycosides, such as gentamicin (G), are indicated in the management of acute exacerbations of CF as well as in the control of chronic infection and the eradication of *Pa* infections. However, parenteral administration of aminoglycosides requires high doses due to their high polarity and, consequently, reduced penetration into the endobronchial space (Mendelman et al., 1985). Aerosolized aminoglycosides, on the contrary, may deliver the drug directly to the site of action and reduce systemic toxicity and side effects, including severe kidney damage and hearing loss (Geller, 2009; Parlati et al., 2009). Interestingly, among aminoglycosides, G has shown the ability to partially restore the expression of the functional protein CFTR (cystic fibrosis transmembrane conductance regulator) in CF mouse models bearing class I nonsense mutations (Clancy et al., 2001; Du et al., 2002; Wilschanski et al., 2000; Wilschanski et al., 2003). In particular, Du and coll. (Du et al., 2002) demonstrated that G was able to induce the expression of a higher CFTR level compared to tobramycin.

Although aerosolized antibiotics were first introduced in therapy in the ‘50s, recently approved products for life-threatening lung infections in CF are limited to solutions for nebulization (TOBI®, Bramitob® and Cayston®). Generally, aqueous solutions for inhalation may deliver low and variable drug amount, are time consuming, difficult in the dose handling, require routine maintenance in order to avoid microbial contamination, and cause drug chemical instability, as well. Dry powder inhalers (DPI) decrease the burden of treatment and offer more freedom to patients as they are breath-actuated, propellent-free and easy to be transported (Khassawneh et al., 2008). DPI containing drugs as micronized powder are able to aerosolize and deliver a metered and high amount of the active principle to the respiratory tract. They seem to be more suitable than liquid nebulizer products for antibiotic pulmonary therapy, which requires larger drug doses compared to bronchodilator or steroidal treatment.

Concerning physico-chemical properties of gentamicin sulfate, some authors pointed out its high hygroscopicity (Della Porta et al., 2010) which can interfere with the production of respirable particle during aerosol generation. In addition, as particles enter the airways, due to the highly humid environment they may be subject to hygroscopic growth, which reduces lung deposition. In order to produce G powders suitable for inhalation, excipients able to reduce the drug water uptake and to enhance powder flow properties need to be considered. As a matter of fact, aminoacids (AAs) are considered to be safe as pulmonary excipients and were recently used to improve aerosolization behavior of several drugs (Ibrahim et al., 2010; Pilcer and Amighi, 2010; Thai et al., ; Wang et al., 2009). Among AAs, L-leucine (leu) shows a hydrophobic side chain which potentially may help to reduce G water absorption. Moreover, in a previous work we demonstrated that leu is able to increase the dispersibility and, consequently, respirability of dry polyphenol powders for inhalation (Prota et al., 2011).

The aim of this study was to develop, by particle engineering via spray drying, inhalable G powders that have satisfying aerodynamic properties and good stability profile for the treatment of *Pa* infections in CF. Microparticles were designed while studying the effect of leu, feed composition and process parameters on particle formation, physico-chemical properties and aerosol performance. Finally, the effect of the produced powders on cell viability and cell proliferation of bronchial epithelial cells bearing a CFTR F508/ F508 mutant genotype (CuFi1) was investigated by MTT and ELISA assays, respectively.

2. Materials and methods

2.1. Materials

Gentamicin sulphate, L-leucine, o-phthalaldehyde and sodium hydroxide anhydrous pellets were supplied by Sigma Aldrich (Milan, Italy). Ethanol 96% (for analysis, USP grade), dichloromethane (for analysis, USP grade), n-hexane (for analysis, Ph Eur grade), were purchased from Carlo Erba Reagents (Milan, Italy). Other solvents and chemicals were of analytical grade. Size 2 gelatine capsules were kindly offered by Qualicaps Europe S.A. (Madrid, Spain). The Turbospin® was kindly donated by PH&T SpA (Milan, Italy).

All the cell culture reagents were purchased from Lonza.

2.2. Powders Preparation

Micronized particles were prepared by spray drying G alone or with leu from different solvents i.e., water, water/ethanol or water/isopropyl alcohol (IPA) mixtures. G and leu were both solubilized in water, then the organic solvent was added under continuous magnetic stirring, reaching a total powder concentration of 5% w/v. The parameters changed in the formulation regarded: i) kind of solvent, ii) water to organic solvent ratio, iii) G to leu ratio (from 100:0 to 8:2 w/w).

The liquid feeds were neutralized with few drops of a 1 M sodium hydroxide solution and dried using a Buchi mini spray dryer B-191 (Buchi Laboratoriums-Tecnik, Flawil, Switzerland) under the following operative conditions: inlet temperature 125 °C for aqueous solutions, 110 °C for hydro-alcoholic solutions, outlet temperature 72-75 °C, drying air flow 500 L/min, aspiration rate 100%, air pressure 6 atmospheres, feed rate 5 ml/min, nozzle 0.5 mm, set in preliminary experiments.

Each preparation was carried out in triplicate. All the spray-dried powders were collected and stored under vacuum for 48 h at room temperature. Production yields were expressed as weight percentage of the final product compared to total amount of the material sprayed. Powders produced were solubilized in distilled water and analyzed in terms of drug content by means of HPLC method described below.

2.3. Powders physico-chemical Properties

2.3.1. G and leu quantification

G quantitative determination by HPLC followed the Pharmacopoeia method (USP 30) as reported elsewhere (Della Porta et al., 2010). Briefly, 25 mg of G raw material was stirred in 25 ml of distilled water until complete dissolution. Five ml of IPA and 4 ml of a previously prepared phthalaldehyde solution were then added to 10 ml of this solution. The solution was stirred and IPA was added to reach a 25 ml volume. Finally, it was heated for 15 min in a water bath at 60 °C, cooled at room temperature, filtered through 0.45 µm filters and analyzed by HPLC at a wavelength of 330 nm (Chromatopac L-10AD system equipped with a Model SPD-10AV UV–vis detector and a Rheodyne Model 7725 injector loop 20 µl, Shimadzu, Kyoto, Japan). Peak areas were calculated with a Shimadzu C-R6A integrator. Phthalaldehyde solution was obtained dissolving 1.0 mg of o-phthalaldehyde in 5 ml of methanol and adding 95 ml of 0.4 M boric acid, previously adjusted with 8 N KOH to a pH of 10.4, and 2 ml of thioglycolic acid. The pH of the resulting solution was adjusted to 10.4 by a 8 N KOH solution. Calibration curves were worked out and proportionality between G concentration and AUC was checked in the range of 5-500 µg/ml.

After adding the phthalaldehyde solution to a sample containing both G and leu, the amino acid reacted with phthalaldehyde, giving rise to a chromophore absorbing at 330 nm, as observed for G, with no interference with G. Calibration curves were worked out for leu, too, and proportionality between leu concentration and AUC was tested in the range of 1-20 µg/ml.

2.3.2. G and leu solubility

G and leu solubility in water and hydro-alcoholic solutions used for spray drying process (pH 7.0 ± 0.1) was evaluated according to USP 31. An excessive amount of powder was introduced into glass vials containing 8 ml of solvents; the samples were stirred and stored at 25 °C for 3 days. After that, samples were centrifuged for 15 min at 3.000 rpm, in order to remove the extra powder required to saturate the solutions. Supernatants were filtered with 0.45 μm filters and the concentration of dissolved G or leu was determined by HPLC method as described before. The solubility measurements were performed in triplicate.

2.3.3. Particle size

Particle size of both raw materials and spray-dried powders was determined using a laser light-scattering granulometer equipped with a micro liquid module (LS 13 320 Beckman Coulter Inc., Fl, USA). In preliminary studies, dichloromethane was chosen as suspending medium among the other chemicals. Samples were suspended in dichloromethane and sonicated for 2 min: few drops of each sample were poured into the small-volume cell to obtain an obscuration between 8 and 12%. Particle size distributions were calculated by instrument software, using the Fraunhofer model. Results were expressed as d_{50} and span defined as $[d_{90}-d_{10}]/d_{50}$, where d_{90}, d_{50} and d_{10} indicate the volume diameters at the 90^{th}, 50^{th} and 10^{th} percentiles respectively.

2.3.4. Scanning Electron Microscopy (SEM)

Morphology of raw materials and microparticles was investigated using a scanning electron microscope (SEM) Zeiss EVO MA10 (Carl Zeiss SMT AG, München-Hallbergmoos, Germany) operating at 14 kV.

166 2.3.5. Bulk and tapped density

167 Bulk and tapped densities of the spray-dried powders were measured as described elsewhere
168 (Sansone et al., 2009). Briefly, powders were loaded into a bottom-sealed 1 ml plastic syringe
169 (Terumo Europe, Leuven, Belgium) capped with laboratory film (Parafilm® "M", Pechiney Plastic
170 Packaging, Chicago, IL, USA) and tapped on a hard bench until no change in the volume of the
171 powder was observed. The bulk and tapped densities were calculated from the net weight of the
172 plastic syringe content divided by the powder volume in the syringe before and after tapping,
173 respectively. Experiments were performed in triplicate.

174

175 2.3.6. Moisture uptake

176 The moisture uptake kinetics of raw materials and spray-dried powders was determined after their
177 removal from the spray-drying chamber. About 20 mg of powder were inserted into an aluminum
178 pan and transferred onto the plate of the balance (MTS Mettler Toledo microbalance, OH, USA) at
179 60% RH and 25 °C. The balance was left open during the experiment and the increase in powder
180 weight was measured each 10 min up to 80 min. Results were expressed as the percentage of weight
181 gained by the sample during the time.

182

183 2.4. Aerodynamic Behaviour Evaluation

184 A first screening of the *in vitro* deposition of the spray-dried powders was carried out using
185 a single-stage glass impinger (SSGI, apparatus A Eur. Ph. 6.0, Copley Scientific Ltd., Nottingham,
186 UK) and the Turbospin® as inhalation device. The Turbospin® is a breath-activated, reusable DPI,
187 working with a single unit capsule. The capsule is vertically inserted into the pulverization chamber
188 and pierced by a needle at the bottom side: the inhaled air creates a turbulence that shakes and
189 twists the capsule, facilitating its empty. The selected device has an optimal resistance rate, able to
190 assure an effective particle deaggregation even with a moderate inspiration potency.

For the SSGI experiments, 30 and 7 ml of distilled water were introduced in the lower and upper stages of the SSGI, respectively. Hard gelatine capsules (size 2) were filled manually with different amounts of spray-dried powder (60-120 mg), according to its bulk density. Then, the capsule was introduced into the Turbospin® and pierced twice. The vacuum pump was operated at a flow rate of 60 l/min for 5 s (Erweka vacuum pump VP 1000 equipped with an electronic digital flowmeter type DFM, Erweka Italia, Seveso, MI, Italy). Each deposition experiment was performed on 3 capsules and repeated in triplicate. Upper and lower parts were washed with 500 ml of distilled water, in order to recover the powder deposited on each stage, the G content of which was evaluated by HPLC as described above. The emitted dose (ED) was gravimetrically determined and expressed as percentage of powder exiting the device *vs* amount of powder introduced into the capsule. The fine particle fraction (FPF), defined as ratio of G recovered from the lower stage of SSGI *vs* total G charged into the capsules, was expressed as a percentage (Sansone et al., 2009).

The powders showing promising aerosolisation properties were also tested by Andersen cascade impactor (apparatus D, Eur. Ph. 6.0, ACI, Westech Instrument Services Ltd., Bedfordshire, UK), adjusted for use at a flow rate of 60 L/min as described elsewhere (Gilani et al., 2005; Seville et al., 2007). The effective cut-off diameters of the modified ACI, provided by the producer, were: Stage -1, 8.6 μm; Stage -0, 6.5 μm; Stage 1, 4.4 μm; Stage 2, 3.2 μm; Stage 3, 2.0 μm; Stage 4, 1.1 μm; Stage 5, 0.54 μm; Stage 6, 0.25 μm. In order to minimize particle bounce, metal impaction plates were dipped into an *n*-hexane solution of SPAN 80 (0.1% w/v) and the solvent was allowed to evaporate, leaving a thin film of SPAN 80 on the plate surface. The ACI was assembled placing a filter paper on the filter stage and the Turbospin® was fitted into a rubber mouth piece attached to the throat. Four hard gelatine capsules (size 2) were filled manually with 120 ± 0.5 mg of sample. Each capsule was introduced into the Turbospin® and pierced twice. The vacuum pump was actuated for 4 s. The powder deposited into the different stages was recovered by plunging each plate and the stage below into distilled water (5-500 ml depending on the stage number). G content

was assessed by HPLC measurements. The emitted dose (ED) was determined as described above for SSGI experiments. The cumulative mass of powder with a diameter lower than the stated size of each stage was calculated and plotted as a percentage of recovered powder *vs* cut-off diameter. The mass median aerodynamic diameter (MMAD) of the particles was extrapolated from the graph, according to the Eur. Ph. 6.0. From the same plot, the fine particle dose (FPD), i.e. the mass of G with a particle size less than 5 μm, and the fine particle fraction (FPF), i.e. the fraction of G emitted from the device with a particle size less than 5 μm, were determined. *In vitro* deposition experiments were performed on three batches with three replicates each.

2.5. Powder stability

Physicochemical stability of G powders dried from hydroalcoholic solutions and containing 15% w/w of leu was assessed after a 6 month storage at 25 °C ± 2 °C/60% RH ± 5% RH in a climatic chamber (Climatic and Thermostatic Chamber Mod. CCP37, AMT srl, MI, Italy), with emphasis on drug content, surface morphology and aerodynamic properties. All measurements were performed in triplicate.

2.6. *In vitro* toxicity

2.6.1 Cell line and culture conditions

CuFi1 cell line, derived from human bronchial epithelium of a CF patient (CuFi1, CFTR ΔF508/ ΔF508 mutant genotype), was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). CuFi1 cells were grown in human placental collagen type VI coated flasks (Sigma Aldrich, Milan, Italy) in bronchial epithelial basal medium, BEBM (Clonetics, Lonza, Walkersville. Inc) supplemented with BPE, hydrocortisone, hEGF, epinephrine, insulin, triiodothyronine, transferrine and retinoic acid (all from Lonza) and penicillin/streptomycin (50 mg/ml). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2**.**

241 For *in vitro* biological studies, the powders were dissolved in sterile water, and immediately
242 administered to the cells.
243
244 2.6.2. Proliferation assay
245 Cell growth was assessed by using a colorimetric bromodeoxyuridine (BrdU) cell
246 proliferation ELISA kit (Roche Diagnostics, Milan, Italy). Briefly, 10 x 10^3 cells were seeded into
247 each coated well of a 96-well plate and left to adhere to the plate. The cells were then treated with
248 increasing concentrations (from zero to 2 µM) of rawG, Get3 and Get3-Leu15 for 24 h. BrdU was
249 added for the final 16 h (10 µM final concentration). At the end of the cell culture period, the
250 medium was removed and the ELISA BrdU immunoassay was performed as described by the
251 manufacturer. The colorimetric reaction was stopped by adding H_2SO_4, and the absorbance at 450
252 nm was measured using a microplate reader (Bio-Rad Laboratories, Milan, Italy).
253
254 2.6.3 Viability assay
255 Cell viability was analyzed using the MTT assay. Briefly, cells were seeded at the density of
256 10 x 10^3/well, left to adhere to the plate and then treated with rawG, Get3 and Get3-Leu15 for 24 h.
257 3-(4,5-methylthiazol- 2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) was added (0.5 mg/ml final
258 concentration) to each well of the 96-well plate and incubated in 37 °C for 4 h. Formazan products
259 were solubilised with 10% Triton X-100, 0.1 N HCl in 2-propanol. Absorbance was determined at
260 595 nm using a microplate reader (Bio-Rad Labaoratories srl, MI, Italy).
261
262 2.7. Statistical analysis
263 Measurements were performed in triplicate, unless differently stated. Values expressed as
264 mean of at least three experiments with three replicates each ± SD. Statistical differences between

the treatments and the controls were evaluated by the Student's *t*-test A (*P* values less than 0.05 were considered statistically significant).

3. Results and discussion

3.1. Manufacturing and characterization of spray-dried powders

Due to its high polarity, G powder as raw material was deliquescent, becoming liquid after 1 hour of exposure to room conditions. In order to reduce powder hygroscopicity and stickiness, G was spray dried alone or with leu as potential flowability enhancer using water or water-co-solvent systems with different dielectric constant (water, water/ethanol or water/IPA mixtures): batches processed from hydro-alcoholic solutions containing ethanol are indicated as Get and those containing IPA as Giso.

Preliminarly, the solubilities of the drug and excipient in the feed systems were determined; G freely soluble in water exhibited the lowest solubility in water/IPA 7/3 (v/v) system, the poor solubility of leu is even lower in water-co-solvent systems (Table 1).

As reported in Table 2, addition of the organic co-solvents into the water feed was extremely helpful in terms of spray drying process yield. In particular, less polar IPA led to higher process yield than ethanol. Batch dried from a 7/3 v/v water-IPA solution showed a 30% increase in yield, compared with powder dried from water, suggesting a reduction in powder cohesiveness and, therefore, a potential enhancement of the aerosolisation properties (Li et al., 2005). Differently, leu addition did not have a linear effect on spray drying yield, especially in hydro-alcoholic solutions (Table 2). HPLC analysis evidenced that the amount of G and leu detected in all produced batches was almost 100% of nominal load, therefore indicating that the spray drying process on the selected conditions neither determined loss nor modified G/leu ratio in the final product. Particle size analysis showed that spray-drying allowed to obtain micronized powders with d_{50} (ranging from 3.6 µm to 4.8 µm) similar for all batches produced (Table 2), with no evident effect of co-solvent and leu content on the particles diameter.

Organic co-solvent had a massive effect on hygroscopicity too (Fig. 1). In particular, by adding 30% v/v of IPA into the aqueous feed, humidity uptake by G powders was reduced from 10.5% (water) to 4.8% (water/IPA) after exposure at room conditions. In the presence of 10% w/w leu, G lost its water avidity (0.9% weight gained after 80 min). These effects may be explained by the addition of the lower-soluble component (leu) into the liquid feeds, able to reach the critical concentration for shell formation as the droplet evaporation progresses during spray-drying process (Vehring, 2008). Such enrichment in leu at the particle surface may slow down G water avidity in agreement with previous observations (Shur et al., 2008) and, potentially, increase powder flowability.

Morphology studies showed an increase in particle corrugation as an effect of leu presence in spray-dried powders. As an example, SEM pictures of particles dried from 8:2 water/ethanol ratio solutions were reported in Fig. 2. As well known, the morphology of spray-dried particles is strongly influenced by the solubility of the components and their initial saturation in the liquid feeds. G, freely soluble in water, led to the formation of spherical particles when spray dried alone (Fig. 2a, G). According to previous observations (Lechuga-Ballesteros et al., 2008), during the co-spray drying process, the saturation of the lower-soluble component (leu) may increase faster than that of hydrophilic one (G), due to the preferential evaporation of alcohol and the associated change in the solvent/co-solvent ratio. This led to the formation of a primary solid shell which collapsed, hence corrugated microparticles were formed. As the relative amount of the less soluble component increased, particle corrugation was more and more evident; particles from almost spherical became raisins like (Fig. 2b, G/5%leu) or irregularly wrinkled (Fig. 2d, G/20%leu). Such surface modification has been shown to be beneficial for particles intended for inhalation (Chew and Chan, 2001): a corrugated surface improves powder dispersibility by minimizing contact areas and reducing interparticulate cohesion and, therefore, corrugated particles disperse better than spherical ones.

By modifying particle shape and corrugation degree, leu influenced powder bulk density too (Table 2). In fact, powders processed from hydro-alcoholic systems showed lower bulk density values than those spray-dried from water (Table 2), whereas leu inclusion up to 15% w/w led to higher density powders. Further increase in leu content up to 20% w/w produced powders with similar or slightly lower bulk density.

As well known, differences in bulk density influence the amount of powder chargeable into the capsules for the inhalation, which shifted from 60 mg for neat G to 120 mg for G/10-20%leu. As a consequence, an important effect on the patient compliance can be achieved in the case of antibiotics such as G requiring the administration of high doses. Previously, a pilot study on effectiveness and toxicity of G administered as dry powder inhaler, (Crowther Labiris et al., 1999) reported that 32 actuations of the device were necessary to emit 160 mg of G nominal dose. In the case of G/10-20%leu DPI, the possibility to charge higher amount of drug into the device allows the administration of 108 mg (G/10%leu) or 96 mg (G/20%leu) of G each time, with a dramatic reduction in the number of actuations required.

3.2. Aerodynamic behavior

The preliminary screening of the powder aerosol performance was carried out by Single Stage Glass Impinger using Turbospin® as inhaler device. Capsules were filled with different amount of dry powder (60-120 mg), depending on its bulk density.

Batches dried from water were hygroscopic, cohesive powders, difficult to insert into and come out from the capsule and with unsatisfying aerodynamic properties (data not shown). In particular, neat G dried from water was a sticky material, unable to be aerosolized.

Results from *in vitro* SSGI deposition experiments for batches different in co-solvent and aminoacid content are reported in Table 3. G spray drying from water/organic co-solvent (e.g.

water/ethanol-based Get2 and Get3, water/IPA-based Giso2 and Giso3) reduced powder cohesivity and enabled the aerosolization process; however, the resulting aerodynamic properties were still not satisfying (FPF less than 15%). The inclusion of leu substantially increased emitted doses (ED up to 99.6% for #Giso2-Leu15) and fine particle fractions (FPF up to 49.4% for #Giso3-Leu15). Taking into account the relative reduction in drug content, further increase in the excipient/drug ratio up to 20/80 w/w did not improve DPI performance. As to the effect of organic co-solvents, the use of IPA led to the best FPF and FPD values. As example, Giso3-Leu15 formulations, containing 15% w/w of leu and obtained from 30% v/v of IPA/water feed, emitted 50.4 mg of fine G after one actuation of the device, compared to a FPD of 44.5 mg of Get3-Leu15, containing the same amount of leu and co-solvent, but processed from ethanol. These results are in agreement with previous studies (Chew and Chan, 2001; Chew et al., 2005; Weiler et al., 2010) evidencing the enhancement of powder aerosol performance as particle surface corrugation goes up to a certain degree; further corrugation enhancement did not improve aerodynamic properties. Plotting FPF values of powder dried from 20% IPA feed versus growing leu amounts (Fig. 3) and in relation to SEM micrographs, a dramatic increase in both particle corrugation and FPF was shown as the leu content enhanced.

On the basis of these interesting preliminary results, powders containing 15 or 20% w/w of leu were analyzed by means of Andersen cascade impactor too, in order to study details of their aerodynamic properties. Results are reported in Table 4. MMAD, FPF and FPD values obtained by ACI deposition studies confirmed the previously observed trend. Capsules charged with 120 mg of powder emitted almost the whole dose from the device after the pump actuation, as indicated by ED values $\geq$ 99.2%. Increase of leu content from 15 to 20% w/w did not enhance the powder aerosol efficiency, whereas a reduction in particles MMAD values as well as a general improvement in powder aerosol performance was observed for batches processed from higher amount of co-solvent, especially IPA (Giso). Among all formulations, Giso3-Leu15 (G/15%leu

366 from 3/7 v/v IPA/water feed) showed very satisfying aerodynamic properties as proved by MMAD
367 of 3.45 μm, FPF 58.1% and FPD of 56.4 mg (Table 4).

368 For a preliminary screening of stability, powders were stored in a climatic chamber for 6
369 months at 25 °C ± 2 °C/60% RH ± 5% RH. During this time, no variation in powder weight was
370 observed, G content remained unaltered and no G degradation product was recorded by HPLC
371 analyses of aged powders. Moreover, in order to evidence possible changes in inhalation
372 performance, ACI studies were repeated on 15% leu powders. Results (Table 4, black rows) showed
373 that ED, FPF and FPD values of aged powders were not significantly different with respect to the
374 fresh ones except for #Get2leu15 showing slightly lower FPD (from 38.8 mg to 33.7 mg). These
375 findings confirmed that G/Leu systems designed are not hygroscopic and are able to preserve a high
376 dispersibility even after 6 month storage.

377

378 3.3. Cytotoxicity *in vitro*

379 In order to establish whether the particle engineering has any cytotoxic or cytostatic effect
380 on bronchial epithelial cells bearing a CFTR F508/ F508 mutant genotype (CuFi1) (Dechecchi et
381 al., 2008; Zabner et al., 2003), CuFi1 cells were treated for 24 h with increasing concentrations
382 (from 0.0002 to 2 μM expressed as G content) of Get3 or Get3-Leu15 powders in comparison to
383 rawG. Results indicated that neither rawG nor its formulations generally inhibited cells viability as
384 determined by MTT assay (Fig. 4B). At concentrations higher than 0.02 μM, a slight but significant
385 decrease in cell survival was detected only for rawG. An interesting observation is that an increase
386 in leu content up to 15%, as in Get3-Leu15, faintly but not significantly decreased CuFi1 viability at
387 concentration ranging from 0.02 to 0.2 μM ($P<0.05$) (Fig. 4B) whereas at 2.0 μM did not. As
388 previously reported (Holt et al., 1985; Prota et al., 2011; Switzer et al., 2009), this effect seems to
389 be related to the leu ability to improve cell proliferation and metabolism of bronchial epithelial CF
390 cells.

Furthermore ELISA BrdU immunoassay evidenced that rawG slightly reduced CF cell growth only at the highest concentration (2 µM, $P<0.01$) (Fig. 4A).

Therefore, particle engineering producing G/leu systems had no cytotoxic or cytostatic effect on CF epithelial lung cells (CuFi1 model), compared to neat rawG, at concentrations up to 2 µM.

4. Conclusions

The engineering process by spray drying and the use of water-co-solvent systems as liquid feed reduced G powder hygroscopicity and stickiness, allowing its aerosolization. Moreover, the addition of small amount of safe excipients, as leu, led to powder with an excellent emitted dose and good aerodynamic properties after actuation of the Turbospin device. In particular, dry powder inhalers containing 15% of leu (Giso 3-Leu 15) was able to deliver almost 100 mg of G with a 58% of FPF after a single actuation. Preliminary stability studies evidenced that dry powders preserved good inhalation performance after a 6 month storage at room conditions. Finally, the engineered particles showed no cytotoxic or cytostatic effect on bronchial epithelial cells bearing a CFTR F508/ F508 mutant genotype.

These findings together with the well known G antibiotic activity and ability to partially restore CFTR expression in class I nonsense mutation, support the use of G/leu DPI as a valid alternative to antibiotics already used in the management of *Pa* infections.

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Table(s)

Liquid feed composition	G mg/ml	Leu mg/ml
Water	Freely soluble	24.2±1.0
Water/ethanol 8/2 (v/v)	519.4±97.0	14.6±0.3
Water/ethanol 7/2 (v/v)	242.0±25.1	10.1±0.5
Water/IPA 8/2 (v/v)	351.8±25.1	11.2±0.5
Water/IPA 7/3 (v/v)	135.9±24.6	9.5±0.2

Table 1- Aquino et al

	Code #	Leu content (%w/w)	Process yield (%)	d_{50} (µm) and () span	Bulk density (mg/ml)		Code #	Leu content (%w/w)	Process yield (%)	d_{50} (µm) and () span	Bulk density (mg/ml)
20% v/v ethanol	**Get2**	0	61.2 ±5.4	4.42 (1.98)	0.13 ±0.02	**20% v/v IPA**	**Giso2**	0	78.0 ±3.8	4.74 (2.10)	0.11 ±0.02
	Get2-Leu5	5	77.6 ±2.2	4.03 (1.55)	0.22 ±0.01		**Giso2-Leu5**	5	73.9 ±0.5	6.19 (1.88)	0.16 ±0.02
	Get2-Leu10	10	58.0 ±3.2	4.58 (2.04)	0.36 ±0.01		**Giso2-Leu10**	10	65.0 ±5.5	4.07 (1.81)	0.29 ±0.01
	Get2-Leu15	15	69.3 ±0.3	4.65 (1.94)	0.35 ±0.02		**Giso2-Leu15**	15	84.6 ±3.3	3.72 (1.58)	0.34 ±0.00
	Get2-Leu20	20	72.6 ±1.1	4.46 (1.88)	0.32 ±0.01		**Giso2-Leu20**	20	77.5 ±0.6	4.82 (1.73)	0.33 ±0.01
30% v/v ethanol	**Get3**	0	74.8 ±2.5	4.01 (1.82)	0.15 ±0.01	**30% v/vIPA**	**Giso3**	0	85.5 ±0.7	4.24 (1.97)	0.19 ±0.02
	Get3-Leu5	5	69.4 ±2.2	4.34 (1.81)	0.24 ±0.02		**Giso3-Leu5**	5	86.6 ±1.2	3.77 (1.36)	0.17 ±0.01
	Get3-Leu10	10	82.5 ±3.1	3.59 (1.57)	0.29 ±0.00		**Giso3-Leu10**	10	85.9 ±0.9	3.69 (1.51)	0.26 ±0.02
	Get3-Leu15	15	68.2 ±4.1	4.16 (1.71)	0.34 ±0.00		**Giso3-Leu15**	15	82.0 ±2.1	3.90 (1.62)	0.34 ±0.01
	Get3-Leu20	20	68.9 ±2.1	4.65 (1.88)	0.31 ±0.01		**Giso3-Leu20**	20	80.8 ±1.3	4.11 (1.90)	0.30 ±0.00

Table 2- Aquino et al

	Code #	Leu content (%w/w)	Charged Dose (mg)	ED (%)	FPF (%)	FPD (mg)		Code #	Leu content (%w/w)	Charged Dose (mg)	ED (%)	FPF (%)	FPD (mg)
20% v/v ethanol	Get2	0	60	95.6 ±1.4	17.3 ±3.8	10.4 ±2.3	**20% v/v IPA**	**Giso2**	0	60	95.8 ±1.9	14.5 ±7.8	8.7 ±4.7
	Get2-Leu5	5	90	98.0 ±0.3	23.7 ±9.2	20.2 ±7.9		**Giso2-Leu5**	5	80	98.0 ±0.2	21.9 ±5.1	16.6 ±3.9
	Get2-Leu10	10	120	99.2 ±0.1	28.9 ±5.2	31.3 ±5.6		**Giso2-Leu10**	10	120	99.4 ±0.1	32.6 ±5.6	35.2 ±6.0
	Get2-Leu15	15	120	99.3 ±0.3	31.0 ±1.5	31.6 ±1.5		**Giso2-Leu15**	15	120	99.6 ±0.2	46.8 ±0.5	47.7 ±0.5
	Get2-Leu20	20	120	99.2 ±0.1	40.8 ±1.5	39.2 ±1.5		**Giso2-Leu20**	20	120	99.3 ±0.3	50.9 ±1.0	48.8 ± 0.9
30% v/v ethanol	Get3	0	60	95.7 ±2.2	18.9 ±4.8	13.4 ±2.9	**30% v/vIPA**	**Giso3**	0	60	90.9 ±7.9	13.4 ±8.5	7.5 ±4.9
	Get3-Leu5	5	80	98.0 ±0.5	14.9 ±1.5	11.4 ±1.1		**Giso3-Leu5**	5	70	97.2 ±0.5	22.3 ±3.0	14.8 ±2.0
	Get3-Leu10	10	100	98.5 ±0.6	38.6 ±5.7	34.7 ±5.1		**Giso3-Leu10**	10	110	99.4 ±1.1	28.8 ±5.0	28.4 ± 5.0
	Get3-Leu15	15	120	98.9 ±0.2	43.6 ±2.7	44.5 ±2.8		**Giso3-Leu15**	15	120	99.1 ±0.3	49.4 ±0.8	50.4 ±0.8
	Get3-Leu20	20	120	99.0 ±0.1	46.5 ±1.5	44.7 ±1.4		**Giso3-Leu20**	20	120	99.2 ±0.0	50.2 ±1.0	48.2 ±0.9

ED, emitted dose; FPF, fine particle fraction; FPD, fine particle dose

Table 3- Aquino et al

	Code #	ED (%)	MMAD (µm)	FPD (mg)	FPF (%)		Code #	ED (%)	MMAD (µm)	FPD (mg)	FPF (%)
20% v/v ethanol	**Get2-Leu15 (t=0)**	99.2 ±0.3	4.2 ±0.3	38.9 ±1.5	39.2 ±1.2	**20% v/v IPA**	**Giso2-Leu15 (t=0)**	99.7 ±0.3	4.0 ±0.1	49.3 ±1.7	46.0 ±2.7
	Get2-Leu15 (t=6 months)	99.4 ±0.3	4.4 ±0.2	33.7 ±2.3	35.4 ±1.5		**Giso2-Leu15 (t=6 months)**	99.3 ±0.2	3.5 ±0.1	50.6 ±2.0	49.0 ±1.8
	Get2-Leu20 (t=0)	99.3 ±0.2	4.1 ± 0.1	40.9 ±2.5	42.8 ±0.7		**Giso2-Leu20 (t=0)**	99.6 ±0.4	4.2 ± 0.1	39.3 ±0.3	42.5 ±0.2
30% v/v ethanol	**Get3-Leu15 (t=0)**	99.5 ±0.3	4.3 ±0.2	47.5 ± 3.9	40.6 ±4.6	**30% v/v IPA**	**Giso3-Leu15 (t=0)**	99.2 ±0.3	3.4 ±0.2	56.4 ±1.1	58.1 ±3.6
	Get3-Leu15 (t=6 months)	99.4 ±0.3	3.8 ±0.3	46.7 ±3.2	44.4 ±1.8		**Giso3-Leu15 (t=6 months)**	99.2 ±0.4	3.3 ±0.2	56.1 ±0.6	52.5 ±0.0
	Get3-leu20 (t=0)	99.2 ±0.3	3.93 ±0.20	41.9 ±2.1	45.3 ±2.0		**Giso3-Leu20 (t=0)**	99.2 ±0.2	3.3 ±0.1	54.7 ±2.2	58.0 ± 0.5

ED, emitted dose; MMAD, mass median aerodynamic diameter; FPF, fine particle fraction; FPD, fine particle dose.

Table 4- Aquino et al

Table(s) captions

Table 1. Gentamicin and L-leucine solubility in liquid feeds used for spray drying at pH 7.0±0.1.

Table 2. Physical characteristics of spray dried particles: liquid fees composition, process yield, particle size and bulk density.

Table 3. Aerodynamic properties of spray-dried powders after single stage glass impinger deposition experiments. All data are shown as mean ± SD of three experiments.

Table 4. Aerodynamic properties of G spray-dried powders containing 15 or 20% w/w leu after Andersen cascade impactor deposition experiments (t=0). Experiments were repeated on powders containing 15% leu w/w after 6 month storage: results are reported in black rows.

Figure(s)

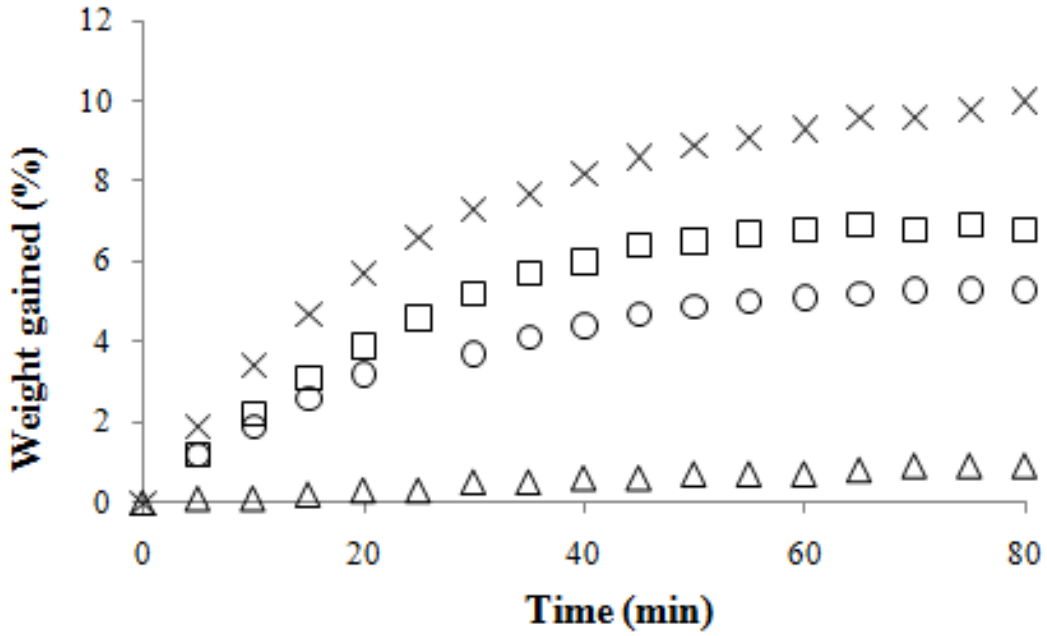

Figure 1- Aquino et al

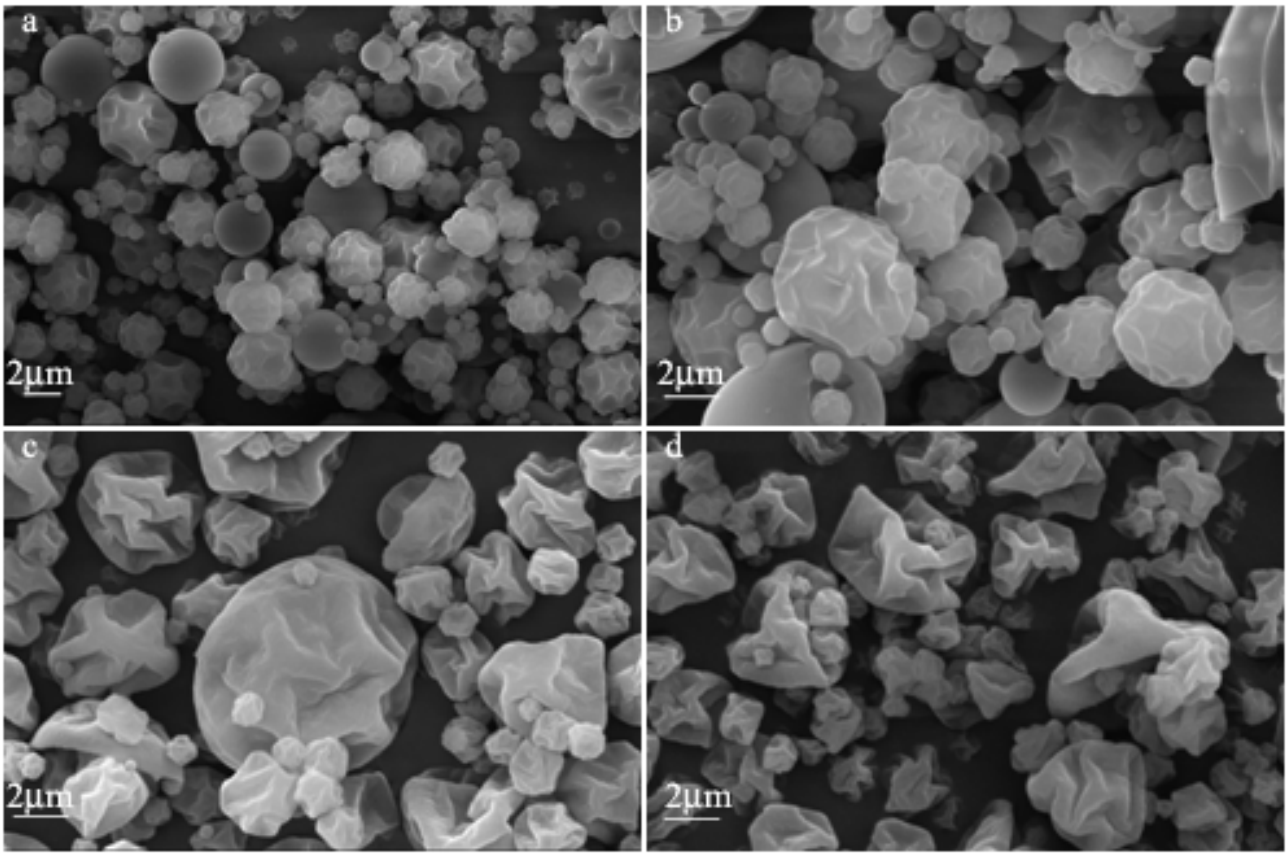

Figure 2- Aquino et al

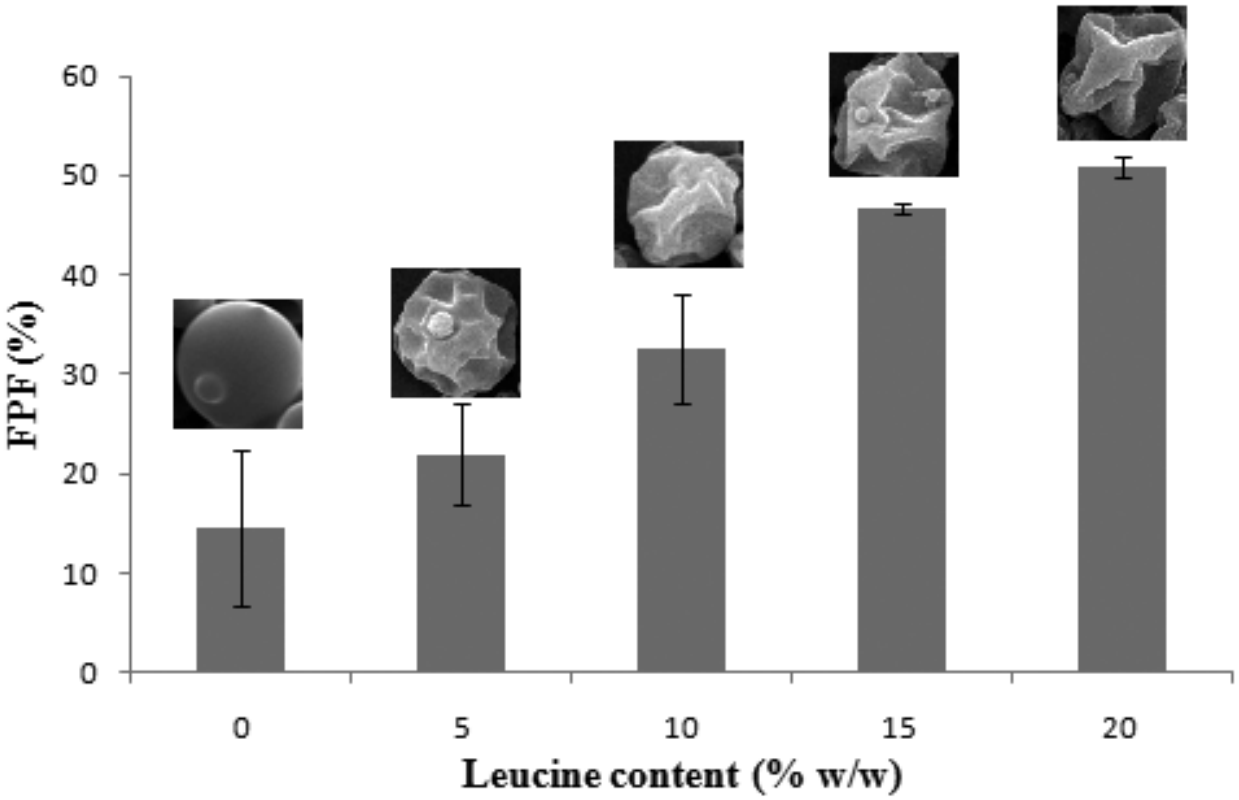

Figure 3- Aquino et al

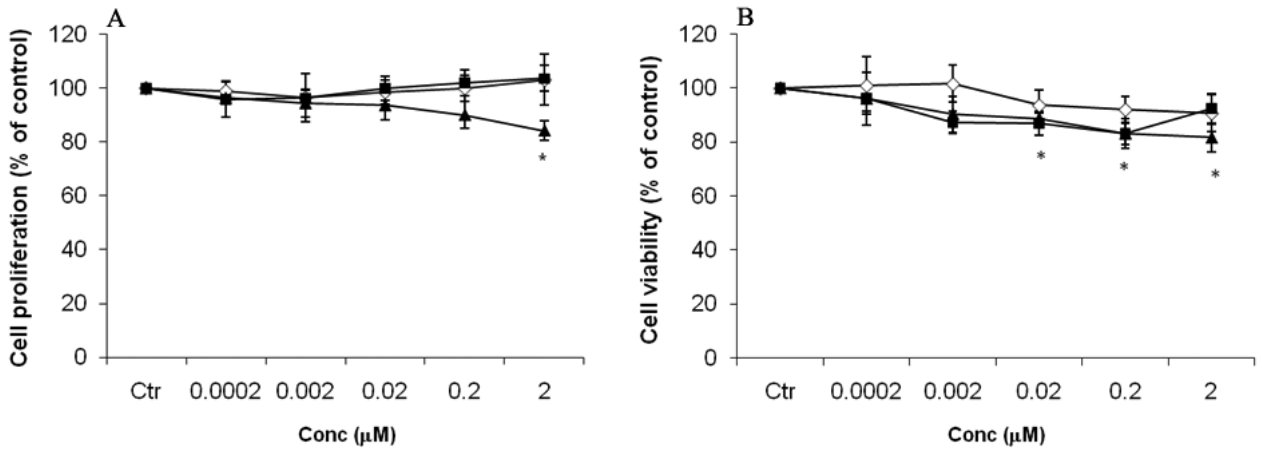

Figure 4- Aquino et al

Figure(s) captions

Fig. 1. Weight gained after 80 min of exposure at room conditions by G raw material (cross), G spray-dried from 7:3 water-ethanol (squares) or water-IPA (circles) v/v systems, and G/10%leu spray-dried from water-IPA 7:3 v/v mixture (triangles).

Fig. 2. SEM pictures of powders dried from water/ethanol 8:2 v/v systems containing: a) G; b) G/5% leu; c) G/10% leu; d) G/20% leu.

Fig. 3. FPF and SEM images of G powders spray-dried from liquid feeds containing 20% IPA and increasing amount of leu.

Fig. 4. Effect of Gentamicin and its DPI formulations on CuFi1 cell proliferation and viability. Cells were treated for 24 h with: raw Gentamicin (rawG, ▲), spray-dried Gentamicin (Get3 ◊) and G co-sprayed with 15%w/w leucine (Get3-Leu15 ■) at concentrations from 0.0002 µM to 2 µM. Cell growth (A) was determined using a colorimetric bromodeoxyuridine (BrdU) cell proliferation ELISA kit. Cell viability (B) was determined by MTT assay. All data are shown as mean ± SD of three independent experiments, each done in duplicate (*P<0.05 and **P<0.01 *vs* control).