Research Article

Synergistic Antibiotic Combination Powders of Colistin and Rifampicin Provide High Aerosolization Efficiency and Moisture Protection

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Received 6 August 2013; accepted 17 September 2013; published online 16 October 2013

Abstract. For many respiratory infections caused by multidrug-resistant Gram-negative bacteria, colistin is the only effective antibiotic despite its nephrotoxicity. A novel inhaled combination formulation of colistin with a synergistic antimicrobial component of rifampicin was prepared *via* co-spray drying, aiming to deliver the drug directly to the respiratory tract and minimize drug resistance and adverse effects. Synergistic antibacterial activity against *Acinetobacter baumannii* was demonstrated for the combination formulation with high emitted doses (96%) and fine particle fraction total (FPF_{total}; 92%). Storage of the spray-dried colistin alone formulation in the elevated relative humidity (RH) of 75% resulted in a substantial deterioration in the aerosolization performance because the amorphous colistin powders absorbed significant amount of water up to 30% by weight. In contrast, the FPF_{total} values of the combination formulation stored at various RH were unchanged, which was similar to the aerosolization behavior of the spray-dried rifampicin-alone formulation. Advanced surface chemistry measurements by XPS and ToF-SIMS demonstrated a dominance of rifampicin on the combination particle surfaces, which contributed to the moisture protection at the elevated RH. This study shows a novel inhalable powder formulation of antibiotic combination with the combined beneficial properties of synergistic antibacterial activity, high aerosolization efficiency, and moisture protection.

KEY WORDS: combination antibiotics; dry powder inhaler; moisture protection; respiratory infection; synergistic antibacterial activity.

INTRODUCTION

Respiratory tract infections caused by multidrug-resistant (MDR) Gram-negative bacteria are a major health problem worldwide (1). Colistin has been increasingly used as the only therapeutic option for the treatment of life-threatening infections caused by MDR pathogens, such as *Acinetobacter baumannii* (2). Colistin, also known as polymyxin E, is a polypeptide antibiotic discovered in late 1940s. Due to high incidence of toxicity in the 1960s, its clinical use waned. But since 1990s, there are renewed interests in colistin because of markedly increasing number of MDR Gram-negative bacteria which are resistant to all other current antibiotics (3). Two forms of colistin are commercially

available: colistin sulfate (referred to colistin below) and colistin methanesulfonate (CMS), which is an inactive pro-drug of colistin (2).

Although colistin has been widely used in the clinic for the MDR respiratory tract infections, parenteral administration of colistin can cause nephrotoxicity, a major dose-limiting factor, in up to 60% of the treated patients (4). Clinical investigations indicate that intravenous colistin may not be very effective, probably due to low drug concentrations in the infected respiratory tract (5). Bacteria reside in the airways where only a small proportion of colistin can reach after parenteral administrations. Therefore, a high dose is required to maintain optimal drug exposure at the infection sites (6). Such a high drug dose *via* systemic administrations may cause adverse effects. Direct delivery of antibiotics to the respiratory tract holds a great promise for achieving higher drug concentrations at the target site with a low systemic exposure (7). A pharmacokinetic study of inhaled colistin solution in cystic fibrosis patients demonstrated significantly higher drug concentrations in sputum ($C_{\max}$ 40 mg/L) for a prolonged period (e.g., 12 h) and negligible systemic drug exposure ($C_{\max}$ 0.15 mg/L) (8).

Nebulization is a complementary treatment for the respiratory infections in hospitals (5). In general, nebulization is tolerated by the patients, but it requires bulky devices, well-trained professional oversight, long administration time, and typically has a low delivery efficiency (9). These issues can be

Electronic supplementary material The online version of this article (doi:10.1208/s12248-013-9537-8) contains supplementary material, which is available to authorized users.

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overcome by dry powder inhalers (DPIs) as exemplified in tobramycin for inhalation, leading to better patient compliance (10). However, inhalation of the high-dose colistin powders may cause coughing in patients (11). Therefore, a dry powder formulation with a high efficiency and reduced dose is desirable for the antibiotic DPI therapy.

Although the bacterial resistance rate for colistin is relatively low currently, there are signs of increasing rates of resistance in clinical isolates (12). Monotherapy of colistin may lead to emergence of resistance (13). Administration of synergistic combination antibiotics is a promising strategy to combat such antibiotic resistance (2). Rifampicin has been frequently used in the clinic in combination with colistin against MDR Gram-negative bacteria (14), and both *in vitro* (15) and *in vivo* synergistic antibacterial activities have been reported (16). However, there is no report on the combination DPI formulation of colistin. In this study, a combination powder formulation consisting of colistin and rifampicin was designed and prepared by co-spray drying in a mass ratio of 1:1 based upon the currently recommended dosage regimens of both antibiotics and *in vitro* synergy data (17). The physicochemical properties, *in vitro* antibacterial activity, and *in vitro* aerosolization performance were evaluated. This study may provide an inhalable combination antibiotic powder formulation with desirable physical properties for treatment of respiratory infections caused by MDR Gram-negative bacteria.

MATERIALS AND METHODS

Chemicals

Colistin sulfate was purchased from Zhejiang Shenghua Biology Co., Ltd (as the supplied formulation; Hangzhou, Zhejiang, China) and rifampicin from Hangzhou ICH Imp & Exp Co. Ltd. (Hangzhou, Zhejiang, China). Acetonitrile and methanol (HPLC grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA) and trifluoroacetic acid from Sigma-Aldrich (Castle Hill, NSW, Australia).

Bacterial Strain

A. baumannii (ATCC 19606) was purchased from the American Type Culture Collection (Manassas, VA, USA), and maintained in tryptone soya broth (Oxoid Australia, Adelaide, SA, Australia) with 20% glycerol at -80°C .

Spray Drying

The feed solutions (6 mg/mL of total solutes) were prepared by dissolving colistin or/and rifampicin (at the mass ratio of 1:1 for the combination) in a co-solvent consisting of ethanol/water (1:1). No excipient was used to generate the formulation. A B-290 mini spray dryer (Büchi Labortechnik AG, Fällwil, Switzerland) was used under the following operating conditions: inlet temperature 60°C ; atomizer setting 700 NL/h, aspirator 40 m^3/h , and feed rate 2 mL/min (18). Spray-dried powder formulations were stored in a desiccator containing silica gel at $20\pm 3^{\circ}\text{C}$ until used.

Minimal Inhibitory Concentrations Against *A. baumannii*

A. baumannii can cause nosocomially acquired respiratory infections (19), and for many MDR strains, colistin is the only

therapeutic option (2). Therefore, it was used to test the activity of our powder formulations. Broth microdilution method was employed to measure the minimal inhibitory concentrations (MICs) against *A. baumannii* (2). Briefly, approximately 10^6 cfu/mL in cation-adjusted Mueller-Hinton broth (CAMHB, Oxoid, Hampshire, England) was used, and sterile drug solutions with a concentration of 5.12 mg/mL were prepared. Drug concentrations of 0, 0.125, 0.25, 0.5, 1, 2, and 4 mg/L were achieved by diluting the stock drug solution with CAMHB. Microplates were incubated at 35°C in a humidified incubator for approximately 20 h. MICs were determined as the lowest concentration without visible bacterial growth.

Particle Sizing

A laser diffraction particle sizer with a Scirocco dry powder module was used to measure the particle size distribution of powder formulations (Mastersizer 2000, Malvern Instruments, Worcestershire, UK). The powder formulations were dispersed through a laser measurement zone by compressed air with a pressure of 4 bar (18). D_{10} (diameter at 10% undersize), D_{50} (diameter at 50% undersize), and D_{90} (diameter at 90% undersize) were calculated from size distribution data. Measurements for each sample were carried out with three replicates.

Particle Morphology

Particle morphology of the powder formulations was evaluated by scanning electron microscopy (SEM, Carl Zeiss SMT AG, Oberkochen, Germany). Formulation powder was spread on a carbon sticky tape and mounted on a SEM stub, followed by sputter coating with gold (15 nm thick) using a K550X sputter coater (Quorum Emitech, Kent, UK). The images were captured at 5 kV.

Dynamic Water Vapor Sorption

Moisture sorption behavior was measured using dynamic vapor sorption (DVS-1, Surface Measurement Systems Ltd., London, UK). Each formulation was exposed to drying at 0% relative humidity (RH) at the beginning of measurement to provide a baseline and then exposed to the various RH ranging from 0 to 90% at 10% RH increments. The environmental RH was increased from 0 to 90% for the sorption cycle and decreased from 90 to 0% for the desorption cycle. Equilibrium moisture content at each testing RH was determined by a dm/dt of 0.002% per minute.

Crystallinity

Crystallinity of the formulations was investigated using a powder X-ray diffraction (PXRD; Shimadzu XRD-6000, Shimadzu Corporation, Kyoto, Japan). $\text{Cu-K}\alpha$ radiation at a generator voltage of 40 kV and a current of 30 mA was employed. The data was collected by the 2θ scan method with 1° as incident beam angle and a scan speed of $2^{\circ}/\text{min}$ in the range of $5\text{--}50^{\circ}$.

X-ray Photoelectron Spectroscopy

X-ray photoelectron spectroscopy (XPS) analysis was performed using an AXIS Ultra DLD Spectrometer (Kratos

Analytical Ltd., Manchester, UK), equipped with a monochromated Al K α source at a power of 140 W (10 mA, 14 kV). A small quantity of each sample was filled into shallow wells of custom-made powder sample holders (20). Charging of the samples during irradiation was compensated by an electron flood gun in combination with a magnetic immersion lens. A reference binding energy of 285.0 eV for the aliphatic hydrocarbon C 1 s component was used to correct for any remaining offsets due to charge neutralization of specimens under irradiation (21). The pressure in the main vacuum chamber during analysis was typically 10^{-6} Pa. Spectra were recorded with the nominal photoelectron detection normal to the sample surface. Note, however, that in the case of powders of random orientations, the microscopic emission angle is ill-defined. As a consequence, the sampling depth might vary between 0 and 5–10 nm depending on the kinetic energy of the measured photoelectrons. The area analyzed on each sample had approx. dimensions of 0.3 $\times$ 0.7 mm. The elemental composition of the samples was obtained from survey spectra (160 eV pass energy) using sensitivity factors supplied by the manufacturer. High-resolution spectra of individual peaks were recorded at 20 eV pass energy which results in a peak width (full width at half maximum) of typically 0.9–1.1 eV for organic polymeric materials.

In order to determine relative fractions of colistin and rifampicin on the surface of the combination particles, reference data were acquired from the two pure compounds. The data obtained from the combination particles were then compared to the reference data and fractions of the pure compounds estimated as follows. The atomic concentration of each of the present elements (C, O, N, and S) in the combination particles was assumed to be a linear combination of the corresponding concentrations in the pure compounds, appropriately scaled and normalized using the number of respective atoms in one molecule of colistin or rifampicin. This method is particularly reliable in cases where the concentrations in the pure compounds are quite different (N and S) but rather unreliable in cases where concentrations are similar (C and O). However, in the case of C and O the high-resolution 1 s spectra for the two compounds are quite different because of the different chemical structures. This allows the surface fractions of colistin and rifampicin in the combination particles to be estimated using curve fitting: the reference spectra of colistin and rifampicin can be used as two model-fit components to calculate optimized curve fits of the spectra of the combination particles. This yields the relative number of either C or O atoms which are present as part of either colistin or rifampicin (20). Scaled using the respective number of C or O atoms in one molecule of the pure compounds, these fractions can be converted to relative molecular fractions of colistin or rifampicin.

Time-of-Flight Secondary Ion Mass Spectrometry

Time-of-flight secondary ion mass spectrometry (ToF-SIMS) experiments were performed using a PHI TRIFT V nanoTOF instrument (Physical Electronics Inc., Chanhassen, MN, USA) equipped with a pulsed liquid metal ^{79}Au primary ion gun, operating at 30 kV energy. Dual charge neutralization

was provided by an electron flood gun and 10 eV Ar $^{+}$ ions. Surface analyses were performed using “unbunched” Au $_1$ instrument settings to optimize spatial resolution. Raw data were collected in positive SIMS mode at a number of locations typically using a 50 $\times$ 50- μm raster area, with 2-min acquisitions (22).

Analyses of colistin and rifampicin standards identified characteristic peak fragments for use in mapping the components in the combination powders. Peaks corresponding to the protonated molecular ion signal for each antibiotic were of low intensity; hence, higher intensity characteristic fragments were used instead. For colistin, the sum of peaks at m/z $\sim$ 30 atomic mass unit (amu) and $\sim$ 86 amu were selected. For rifampicin, m/z $\sim$ 99 amu was selected. Sample spectra and images were processed using WincadenceN software (Physical Electronics Inc., Chanhassen, MN, USA).

In Vitro Aerosolization Performance

A pharmacopeial method of multi-stage liquid impinger (Apparatus C, British Pharmacopeia 2012, Copley, Nottingham, UK) with a USP induction port (USP throat) was used to determine *in vitro* aerosolization performance. Each formulation was stored in 60% RH at 20°C for 24 h prior to dispersion. Dispersion was performed in a controlled environment cabinet: temperature, 20 $\pm$ 3°C and relative humidity, 60 $\pm$ 3%. Each formulation ($\sim$ 10 mg) was filled into hydroxypropyl methylcellulose capsules (size 3, Capsugel, Peapack, NJ) and dispersed through an Aerolizer® device. A standard pharmacopeial dispersion procedure was conducted for 2.4 s at an air flow rate of 100 L/min allowing 4 L of air pass the inhaler (23) with a pressure drop of approximately 4 kPa (24). The cutoff diameters for stages 1–4 of the liquid impinger at 100 L/min were 10.4, 4.9, 2.4, and 1.2 μm , respectively (23). The Aerolizer® was used here as a model device because it is a commercially available inhaler with a low resistance (25,26). Three replicates were carried out for each formulation. Drug deposited on capsule, inhaler, USP throat, stages 1–4, and filter was collected using water for colistin alone and using the co-solvent (acetonitrile/water=3:7) for rifampicin and the combination. The emitted dose was calculated as a percentage of drug released from the capsules and inhaler device. The fine particle fraction total (FPF $_{\text{total}}$) was calculated as the percentage mass of drug particles with an aerodynamic diameter smaller than 5 μm in the aerosol relative to the total recovered drug.

Drug Quantification

Drug concentration was determined using a validated high-performance liquid chromatography method modified from Zhou *et al.* (27) (HPLC, Model LC-20, Shimadzu, Kyoto, Japan) with a PhenoSphere-Next 5 μm C18 150 $\times$ 4.60 mm column (Phenomenex, Torrance, CA) at 214 nm (SPD-20A UV/VIS detector, Shimadzu). The mobile phase consisted of 0.05% trifluoroacetic acid in Milli-Q water (A) and methanol (B). The gradient program was set as: 30% B to 80% B in 15 min, then 80% B to 30% B in 5 min with a flow rate of 1 mL/min. A calibration curve (0.01–1 mg/mL) was prepared for colistin (in water) and for rifampicin and

Table I. Particle Size (Mean $\pm$ SD, $n=3$) and MICs Against *A. baumannii* ATCC 19606 of the Formulations

	D_{10} (μm)	D_{50} (μm)	D_{90} (μm)	MIC (mg/L)
Colistin supplied	1.2 $\pm$ 0.1	7.2 $\pm$ 0.2	21.0 $\pm$ 1.1	0.50
Colistin spray-dried	0.8 $\pm$ 0.1	1.4 $\pm$ 0.1	2.5 $\pm$ 0.1	0.50
Rifampicin supplied	4.2 $\pm$ 0.4	23.6 $\pm$ 2.5	125.2 $\pm$ 13.2	2.00
Rifampicin spray-dried	1.1 $\pm$ 0.1	2.5 $\pm$ 0.1	5.2 $\pm$ 0.1	1.00
Combination co-spray-dried	1.0 $\pm$ 0.1	1.8 $\pm$ 0.1	3.1 $\pm$ 0.2	0.25

combination (in acetonitrile/water, volume ratio 3:7). The calibration curves were linear in the concentration range ($R^2>0.999$).

Effect of Humidity on Aerosolization

Effect of humidity on aerosolization of the spray-dried formulations was examined by storing the formulations at the

RH of 45%, 60%, and 75%. The powder formulations were spread in an open-top plastic container (3 cm in diameter) as thin as possible and placed in the environment-controlled chamber of DVS unit for 24 h at each selected RH and 20°C. Based on the DVS data, 24-h storage is more than sufficient for equilibrium in water sorption. After storage, the aerosolization performance of each powder was evaluated by a multi-stage liquid impinger as described above in an

Fig. 1. SEM images of: **a** supplied colistin, **b** spray-dried colistin, **c** supplied rifampicin, **d** spray-dried rifampicin, and **e** co-spray-dried combination particles

environment-controlled chamber at the corresponding storage RH.

Statistical Analysis

One-way analysis of variance with a Tukey *post hoc* analysis was used for statistical analysis with probability values of less than 0.05 considered as significant difference.

RESULTS

MICs

There was no deterioration in MICs against *A. baumannii* ATCC 19606 for the colistin and rifampicin-alone formulations after spray drying (Table I). An apparent synergy was found as the combination formulation had an MIC of 0.25 mg/L, which was lower than that of either the colistin spray dried alone (0.5 mg/L) or the rifampicin spray dried alone (1 mg/L).

Particle Size

D_{50} of the supplied colistin and rifampicin powders was 7.2 and 23.6 μm , respectively, which may not be suitable for inhalation (Table I). After spray drying, D_{50} values were reduced to 1.4 μm for the colistin alone, 2.5 μm for the rifampicin alone, and 1.8 μm for the combination. D_{90} values of the spray-dried formulations were smaller than 5.2 μm , demonstrating the majority of particles were within the inhalable range.

Particle Morphology

The supplied colistin and rifampicin particles exhibited irregularly angular shapes in the SEM images (Fig. 1). Typically, the majority of supplied colistin particles had particle sizes in the range of 5–15 μm and the supplied rifampicin in the range of 20–60 μm , which were in good agreement with the particle sizing data (Table I). After spray drying, the colistin alone particles possessed near-spherical shapes with corrugated surfaces; while the spray-dried rifampicin particles had wrinkled and flaked shapes. The spray-dried combination particles had similar shapes to the spray-dried rifampicin.

Crystallinity

The supplied colistin powder formulation was amorphous without any crystalline peak in the XRD diffractogram, but the supplied rifampicin was crystalline (Fig. 2). All spray-dried powder formulations showed amorphous XRD patterns. The amorphous forms of spray-dried formulations were not changed after storage at 60% RH and 25°C for a month (data not shown), indicating the physical stability during storage.

XPS

Table II presents the elemental compositions of the two pure compounds, colistin and rifampicin, as well as that of the combination powder. The compositions obtained for the pure compounds agree quite well with their respective molecular

Fig. 2. Powder X-ray diffraction patterns of the antibiotic powder formulations

structures. The slightly higher concentration of carbon in both cases is very common in surface analysis of organic and polymeric materials, and is most likely due to the presence of adventitious carbon.

The concentrations measured in the case of the combination powder, particularly those for N and S, are much closer to the values of rifampicin than those of colistin. For example, in the case of S which is only present in colistin but not in rifampicin, the concentration of S was markedly reduced when colistin was co-spray-dried with rifampicin. This indicates that XPS detected significantly more rifampicin at the surface than colistin. Using the methods described in the “Materials and Methods” section, we estimated the molar fractions of the two compounds (see Supplements). The average molar fraction of rifampicin on the combination particle surface was 95% ($\pm 2\%$) using various methods, confirming that the surface of the combination particles was enriched in rifampicin.

ToF-SIMS

In Fig. 3, the mapping of rifampicin signals (red) and colistin signals (blue) are shown for an area of 50×50 μm . As ToF-SIMS only detects elemental information from the uppermost one to two molecular layers on surface, the data suggested the particle surface of the combination formulations was dominated by rifampicin. However, such coverage of rifampicin on the particle surfaces may not be complete, supported by the observation of small quantity of blue colistin signals. These findings are consistent with the XPS data revealing the presence of a smaller proportion of colistin on the combination particle surfaces.

Table II. Elemental Compositions Determined by XPS Expressed as Relative Atomic Concentrations (in Percent)

	Colistin	Rifampicin	Combination
C	59.2	72.3	71.2
N	16.9	6.0	7.5
O	21.6	21.7	21.0
S	2.4	–	0.3

Fig. 3. Distribution of colistin (blue) and rifampicin (red) on the surface of co-spray-dried combination particles measured by ToF-SIMS (scale bar represents 10 μm)

Aerosolization Performance

Aerosolization performance of the supplied colistin and rifampicin formulations was not measured because their particle sizes were beyond the inhalable range (Table I). All the spray-dried formulations achieved high emitted doses of above 90% (Fig. 4a). The spray-dried colistin formulation had the lowest emitted dose values of 91.0%. There was no significant difference in the emitted dose between rifampicin (95.7%) and colistin (95.9%) in the combination formulation, or between the spray-dried rifampicin and combination formulations ($p > 0.05$). The combination formulation had high FPF_{total} values of 92.5% for colistin and 91.6% for rifampicin. There was no significant difference in FPF_{total} between the spray-dried rifampicin and combination formulations ($p > 0.05$), but the spray-dried colistin formulation had a significantly lower value of 80.0% ($p < 0.05$).

No significant difference was found in the drug deposition on each stage between colistin and rifampicin in the combination formulation ($p > 0.05$), indicating a uniform distribution of two drugs. The spray-dried colistin formulation has significantly greater deposition values on inhaler and throat but significantly lower on filter ($p < 0.05$), which contributed to lower emitted dose and FPF_{total} compared to the spray-dried rifampicin and combination formulations (Fig. 4). Drug deposition of the spray-dried rifampicin formulation was lower on stage 4 but higher on the filter than the combination powder ($p < 0.05$), resulting in no significant difference in the FPF_{total}.

The drug deposition data on filter ($< 1.2 \mu\text{m}$) showed a proportion of submicron particles present in the powder aerosol. Ultra-fine particles ($< 100 \text{ nm}$) may be exhaled due to negligible inertial impaction and sedimentation, but deep slow inhalation or holding breath will aid its deposition in lungs by allowing more time for diffusion (28,29).

Dynamic Vapor Sorption

The spray-dried formulation of colistin alone absorbed significant amounts of water at the elevated RH (Fig. 5). The

Fig. 4. **a** Aerosolization performance and **b** drug deposition of the spray-dried antibiotic powder formulations from the Aerolizer device (error bars represent standard deviation, $n=3$)

water absorption of spray-dried rifampicin and combination formulations was lower than that of the spray-dried colistin. The water absorption isotherm of the combination formulation was in fact between those of the spray-dried colistin and rifampicin alone. All samples showed a sorption hysteresis

Fig. 5. Dynamic water sorption behavior of the spray-dried antibiotic powder formulations

due to slower escape of water molecules during desorption from the invaginations of particles (30). The mass change was near 0% at the end of each cycle for all formulations, indicating the water sorption behavior was reversible and no moisture-induced recrystallization has occurred.

Effect of Humidity on Aerosolization

Given the spray-dried formulations can absorb significant amounts of water (Fig. 5), the effect of storage humidity on the aerosolization of spray-dried formulations was investigated. For the spray-dried colistin-alone formulation (Figs. 6a and 7a), there was no significant difference in the emitted dose and FPF_{total} between the powders stored in 45% and 60% RH ($p>0.05$). However, when the RH was increased to 75%, the emitted dose and FPF_{total} were significantly reduced to 74.3% and 63.2%, respectively ($p<0.05$). The formulations stored in 75% RH had depositions higher on the inhaler and stage 3 but substantially lower on stage 4 and filter ($p<0.05$), leading to an overall reduction in the emitted dose and FPF_{total}.

For the spray-dried rifampicin alone (Figs. 6b and 7b) and combination formulations (Figs. 6c, d, 7c, and d), there was no significant difference in the emitted dose, FPF_{total}, or

drug deposition on each stage between the powders stored at 45%, 60%, and 75% RH ($p>0.05$).

DISCUSSION

Although currently the resistance of MDR Gram-negative bacteria to colistin is still low, colistin-resistant isolates have been increasingly reported (31). Combination therapy of antibiotics is a well-accepted clinical practice to provide synergistic bacterial killing and to minimize drug resistance. Rifampicin has been most frequently used clinically with colistin *via* parenteral routes against MDR Gram-negative bacteria, and the combination therapies were well tolerated by the patients without severe adverse effects (32). An inhaled formulation of such combination may offer additional benefits of higher local drug concentrations over the parenteral administrations for treatment of respiratory infections. The DPI product of CMS (Colobreathe®) has recently been approved in Europe, but there is no marketed combination antibiotic DPIs available worldwide. In this study, the co-spray-dried combination powder has demonstrated a synergistic antimicrobial effect against *A. baumannii* (Table I). Our recent *in vitro* pharmacokinetics/pharmacodynamics study has demonstrated that the combination therapy minimizes colistin resistance

Fig. 6. Effect of relative humidity on aerosolisation performance of **a** spray-dried colistin-alone formulation, **b** spray-dried rifampicin alone, **c** colistin in the co-spray-dried combination; **d** rifampicin in the co-spray-dried combination (error bars represent standard deviation, $n=3$)

Fig. 7. Effect of relative humidity on drug aerosol deposition of **a** spray-dried colistin-alone formulation, **b** spray-dried rifampicin alone, **c** colistin in the co-spray-dried combination, **d** rifampicin in the co-spray-dried combination (error bars represent standard deviation, $n=3$)

against *A. baumannii* (33). Future studies are warranted to examine *in vivo* efficacy of the inhalable combination formulations.

The most common side effects of inhaling colistin (or CMS) powders are throat irritation and cough (11), which are indicated as “very common” for the commercial products of Colobreathe® (34). When patients use Colobreathe, a capsule with a high CMS dose of 125 mg is inhaled *via* a Turbospin inhaler. Inhalation of high doses of CMS powder may contribute to the cough and throat irritation. However, for the combination formulation developed in this study, very few drugs (<1% of the recovered dose) were deposited on the USP throat during aerosolization *in vitro*. This is attributed to the high aerosolization efficiency, and thus the aerosol has a very small proportion of large agglomerates that are more subjected to inertial impaction than fine particles. Although the *in vitro* data may not precisely reflect the *in vivo* deposition, it suggests a minimized throat irritation due to impaction compared to the traditional carrier-based DPIs. It was suggested that reduced drug doses and fewer numbers of repeating inhalation may minimize the severity of coughing (11). For the present combination formulation, the MIC data indicated that 1/4 of the colistin dose in the combination with rifampicin may offer the same antibacterial effect as colistin alone. The benefits of

reduced colistin dose can be further strengthened by a high aerosolization efficiency of the present combination powder formulation. Traditional DPI formulations containing jet-milled drugs generally have only moderate or low aerosolization efficiency due to high surface energy of particles generated during the milling process (35). For example, a moderate FPF value of ~40% was reported when jet-milled colistin powders were aerosolized *via* a Cyclohaler which has the same geometry as the Aerolizer (36). In contrast, the FPF_{total} of the combination powder formulation here was 92% with emitted doses of 96% *via* the Aerolizer. It is well established that corrugated surfaces improve the aerosolization efficiency of micro-sized particles by reducing contact area (37). Both wrinkled shape and hydrophobic surface nature of the combination particles may contribute to its high aerosolization performance.

In recent studies of the colistin-alone powder formulations, relatively high FPF values of 59–67% of the loaded dose were achieved *via* a Twincer™ device. However, the mass losses (drug deposited on the device and USP induction port) were 26.3–29.1% for these jet-milled CMS powder formulations with addition of 2 mg coarse lactose as a device sweeper (38,39). In general, the jet-milled fine powders of most APIs have poor flow, fluidization, and aerosolization

(40), due to the high surface energy created during the milling (41). Coarse lactose carriers are usually added to improve the device emptying (36,42). For the DPIs with high drug dose of antibiotics (usually >10 mg), addition of carriers limits the drug mass that can be administered through one inhalation. The present combination formulation exhibited high emitted doses >90% with less than 1% of drug deposited on the USP induction port without addition of coarse lactose *via* the Aerolizer *in vitro*, which indicates prominent flow and fluidization properties with the plausibility of reduced number of repeating inhalations.

Depositions in the stage 4 and filter of the two drugs in the combination powder are different to that of the drug-alone formulations. This is due to the difference in particle size distribution and particle density. A uniform deposition of the two drugs in the combination was measured on each stage of the impinger (Fig. 4b), demonstrating a homogenous distribution of the two antibiotics achieved in the powder and probably at the particle level (Fig. 3) (43). This feature is beneficial for combination therapy because due to the different pharmacokinetics of combination antibiotics, drug concentration profiles in the infection site (i.e., respiratory tract) can be very different after parenteral administration of two antibiotics. The synergistic antibacterial activity could be compromised because the designed optimal ratio of drug concentrations may not be reached or maintained in the infection site with parenteral administration. By incorporating two drugs in a particle, they are able to simultaneously reach the target infection sites in the respiratory tract with the designed ratio, thereby maximizing the synergy.

One of the major concerns to produce inhalable drug particles by spray drying is their physical and chemical

instability as generally amorphous or partly amorphous drug powders are generated in the process and such powders may recrystallize during storage. The present co-spray-dried combination formulations were physically stable during storage at 60% RH and 20°C, as shown in the PXRD results and the DVS data (non-reversible water absorption for crystallisation was not detected). Moreover, the contents of both drugs were within 94–101% range after a 1-month storage, demonstrating its short-term chemical stability.

An unexpected important finding is that co-spray drying with rifampicin protects the aerosolization of hygroscopic colistin powder from moisture. The amorphous spray-dried colistin alone powder can absorb significant amounts of water up to 30%wt shown by the DVS data. The FPF_{total} was reduced substantially from 80.0% when stored at 60% RH to 63.2% at 75% RH due to the hygroscopicity of colistin particles (Fig. 6a). The particles even fused together when exposed to the extreme RH of 90% and were unable to be aerosolized (Fig. 8). However, after co-spray drying with rifampicin, both the emitted dose and FPF_{total} were unchanged when the RH increased from 45% to 75%, and no particle fusion was observed after exposure to 90% RH. Given the RH had no effect on the aerosolization performance of the spray-dried rifampicin formulations, we hypothesize that rifampicin migrates to and dominates the particle surfaces during drying process. A co-solvent system consisting of ethanol and water was used to dissolve the two drugs with different water solubilities. During drying, more volatile solvent of ethanol evaporates faster from the droplet. Consequently, hydrophobic rifampicin precipitates first on the particle surfaces (44). The fact that particle morphology of the combination is the same to that of rifampicin alone is evident rifampicin contributes mainly to the shell

Fig. 8. SEM images of the spray-dried antibiotic formulations after exposure to RH 90% during DVS measurement: **a** supplied colistin formulation, **b** spray-dried colistin formulation, **c** spray-dried rifampicin, and **d** co-spray-dried combination formulation

forming. The XPS and ToF-SIMS have respectively provided quantitative and qualitative results that suggested the outmost surfaces of combination particles were rich in rifampicin. The enrichment of hydrophobic rifampicin on the particle surfaces inhibits the interaction between water and colistin when exposed to high RH, thus, prevents the deterioration of aerosolization and the fusion of particles. Modification of hydrophobicity of the particle surface has a possibility to alter the pharmacokinetic profile of colistin, which deserves further PK investigation.

CONCLUSIONS

This study has examined a novel inhalable combination formulation of colistin powder with a synergistic antibacterial component of rifampicin. The combination formulation has a remarkably high aerosolization performance with the emitted dose and FPF_{total} over 90% of the recovered dose. Co-spray drying the hygroscopic colistin with the hydrophobic rifampicin has protected the powder aerosolization from moisture. This effect was ascribable to the enrichment of rifampicin on the particle surfaces. To the authors' knowledge, this is the first report of a synergistic antibacterial combination formulation with high aerosolization efficiency and moisture protection, which has great potential to offer better therapeutic effects with much reduced colistin doses and adverse effects.

ACKNOWLEDGMENTS

HKC acknowledges the Australian Research Council (ARC, grant DP120102778 and DP0985367) for the financial support of this study. JL is a National Health and Medical Research Council (NHMRC) Senior Research Fellow. QTZ is supported by an NHMRC Early Career Fellowship. The authors are grateful for the scientific and technical assistance of the Australian Microscopy & Microanalysis Research Facility at the Australian Centre for Microscopy and Microanalysis, The University of Sydney and the Ian Wark Research Institute, University of South Australia. Capsugel Australia is acknowledged for the kind donation of the capsules.

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