Inhaled combination powders for respiratory system infections and disorders

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**INHALED COMBINATION POWDERS
FOR RESPIRATORY SYSTEM
INFECTIONS AND DISORDERS**

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SCHOOL OF PHYSICAL AND MATHEMATICS SCIENCES

2017

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A thesis submitted to the Nanyang Technological University in
partial fulfilment of the requirement for the degree of
Doctor of Philosophy

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Abstract

The use of inhaled or aerosolized drugs to treat respiratory conditions offers the distinct advantages of rapid delivery of drugs to the site of action (i.e. respiratory airways) and bypassing the first-pass effect. Due to the localized drug effects and avoidance of absorption barrier, inhaled drugs can therefore be applied in much lower doses than is possible with oral or parenteral administration, thereby minimizing severe adverse effects. Since dry power inhalers (DPIs) have the advantages of higher delivery doses, enhanced formulation stability and improved patient compliance as compared to the other inhalation delivery platforms, such as nebulizers and pressurized-metered dose inhalers (pMDIs), significant efforts have been devoted to develop novel DPI formulations. More recently, there has been a trend towards the use of combination therapy in inhaled drug delivery. This could potentially contribute to the convenience of drug administration as well as confer synergistic drug interactions (where the effect of the combination treatment is greater than the sum of the single-drug treatments) to the formulations, leading to better treatment adherence and clinical outcomes. Combining two or more therapeutic agents that have complimentary interactions into a single inhaled formulation represents an incremental innovation that has extended the range of therapeutic options for treating of respiratory system infections and disorders.

Hence, this thesis seeks to develop novel DPI combination formulations that have improved efficacies over the single inhaled species. In order to evaluate the performance of a DPI combination product, the DPI combination formulations developed and studied in this thesis are characterized in terms of their physicochemical properties, tested for their in vitro aerosol performance

and assessed on that in-vitro antimicrobial activity or in-vitro inflammatory mediator response.

List of Publications

Journal Papers

1. **Lee, S. H.**; Teo, J.; Heng, D.; Zhao, Y.; Ng, W. K.; Chan, H.-K.; Tan, R. B. H., Steroid-Decorated Antibiotic Microparticles for Inhaled Anti-Infective Therapy. *Journal of Pharmaceutical Sciences* 2014, 103 (4), 1115-1125.
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3. **Lee, S. H.**; Teo, J.; Heng, D.; Ng, W. K.; Zhao, Y.; Tan, R. B. H., Tailored Antibiotic Combination Powders for Inhaled Rotational Antibiotic Therapy. *Journal of Pharmaceutical Sciences* 2016, 105 (4), 1501-1512.
4. **Lee, S. H.**; Heng, D.; Vanessa, J. X.; Chan, K. P.; Ng, W. K.; Zhao, Y.; Chan, H.-K.; Tan, R. B. H., Inhaled Non-Steroidal Natural Alternatives for Anti-Inflammatory Combination Therapy. Submission to *International Journal of Pharmaceutics*.

Conferences

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- Winning entry for Grand prize of ISPE (International Society for Pharmaceutical Engineering) Singapore Student Poster Competition 2015

2. **Lee, S. H.**; Teo, J.; Heng, D.; Zhao, Y.; Ng, W. K., Re-engineered medical therapeutics: A novel inhaled multi-pronged attack against respiratory bacteria. Poster presented at *2015 ISPE Annual Meeting, Philadelphia, USA*, 8-11 November 2015.

- As the Singapore representative (invited by ISPE) to present this work at the ISPE International Student Poster Competition

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Abbreviations

AIDS	Acquired immune deficiency syndrome
AM	Artificial mucus
AMB	Ambroxol hydrochloride
ARIs	Acute respiratory infections
At	Atomic
ATCC	American Type Culture Collection
BAL	Bronchoalveolar lavage
BET	Brunauer–Emmett–Teller
BP	Beclomethasone dipropionate
BSL-2	Biosafety level 2
CAP	Community-acquired pneumonia
CB	Coomassie blue
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CFU	Colony forming unit
CIP	Ciprofloxacin hydrochloride
CLSI	Clinical and Laboratory Standards Institute
CO ₂	Carbon dioxide
COL	Colistin sulphate
COPD	Chronic obstructive pulmonary disease
D ₁₀	Volume diameter under which 10% of the sample resides
D ₅₀	Volume median diameter
D ₉₀	Volume diameter under which 90% of the sample resides
DNA	Deoxyribonucleic acid

DNase	Deoxyribonuclease
DOTS	Directly Observed Therapy Short-course
DPIs	Dry powder inhalers
DPPC	Dipalmitoylphosphatidylcholine
DTPA	Diethylenetriamine pentaacetic acid
DVS	Dynamic vapour sorption
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FDA	Food and Drug Administration
FESEM	Field emission scanning electron microscopy
FEV ₁	Forced expiratory volume in 1 second
FPF	Fine particle fraction
FPF (emitted)	Emitted fine particle fraction
GAT	Gatifloxacin hydrochloride
GI	Gastrointestinal
GOLD	Global Initiative for Chronic Lung Disease
GSD	Geometric standard deviation
HFA	Hydrofluoroalkane
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
HPMC	Hydroxypropyl methylcellulose
ICS	Inhaled corticosteroids
IE	Insufficient evidence
IL-1 β	Interleukin-1 beta
IL-6	Interleukin-6
KCl	Potassium chloride

LABA	Inhaled long-acting β_2 -agonist
LAMA	Long-acting anti-muscarinic antagonist
LEU	Leucine
LOD	Limit of detection
MDR	Multi-drug resistant
MEM	Meropenem trihydrate
MHB	Mueller Hinton Broth
MIC	Minimum inhibitory concentration
MMAD	Mass median aerodynamic diameter
MOC	Micro orifice collector
MSLI	Multi stage liquid impinger
NA	Not applicable
NaCl	Sodium chloride
NGI	Next generation impactor
PIPS	Polymerization-induced phase separation
pMDIs	Pressurized metered dose inhalers
pXRD	Powder X-ray diffraction
RESS	Rapid expansion of supercritical solution
RH	Relative humidity
RI	Refractive index
RIF	Rifampicin
S1	Stage 1
S2	Stage 2
S3	Stage 3
S4	Stage 4
SAA	Supercritical assisted atomisation

SAS	Supercritical antisolvent
SABA	Short-acting bronchodilator β_2 -agonists
SAMA	Short-acting anti-muscarinic antagonists
SCF	Supercritical fluid
SD	Spray-dried
SEDS	Solution enhanced dispersion by supercritical fluids
SSI	Supercritical solvent impregnation
TB	Tuberculosis
TFA	Trifluoroacetic
TGA	Thermogravimetric analysis
TNF- α	Tumor necrosis factor alpha
USP	United States Pharmacopeia
WHO	World Health Organization
Wt	Weight
XPS	X-ray photoelectron spectroscopy

Chapter 1

Introduction

1.1. The human respiratory system

The human respiratory system is a group of tissues and organs working together to enable the exchange of gases (oxygen and carbon dioxide) between the body cells and external environment. The human respiratory system is specially structured, both anatomically and functionally, to allow the inspired gas to reach the most distal areas of lung for gas exchange with the blood. Anatomically, the human respiratory system can be divided into two major parts: the upper respiratory tract that consists of nose (nasal cavity), mouth (oral cavity), pharynx (throat) and larynx (voice box), and the lower respiratory tract that consists of trachea (windpipe), bronchi, bronchiole, alveolar duct and alveoli (Figure 1.1.) [1].

Figure 1.1. Schematic of the respiratory system displayed by the upper and lower respiratory tract region. Reproduced with permission from Ref. [1]. Copyright 2012 Springer

Inhaled gas or air first enters human respiratory system through mouth and nose, passing down the pharynx and larynx into the airways that consists of a series of branching tubes which are getting narrower, shorter and more numerous at each branching as they penetrate deeper into the lung. Trachea is the largest airway and it divides into two primary bronchi (the left and right bronchus) that carry air into lung. Within the lungs, the bronchi themselves sub-divide dichotomously into smaller bronchi (known as secondary and tertiary bronchi) which further divide into bronchioles, and then divide again into terminal bronchioles. Each terminal bronchiole continues to divide to form respiratory bronchioles which terminate into tiny collections of air sacs called alveoli. The alveoli are surrounded by a dense network of tiny blood vessels called capillaries to facilitate gas exchange between air and blood [2, 3].

From a functional point of view, the human respiratory system can be categorized into conducting and respiratory zone. The conducting zone of respiratory system is not involved in the gas exchange but carries air to the site of gas exchange (i.e. respiratory zone). On the other hand, respiratory zone is found deep inside the lungs where oxygen from inhaled air is exchanged for carbon dioxide from the blood [4]. The tracheobronchial tree derived by Weibel [5] describes the human lung structure as a tree where the airways between the trachea and alveolar sacs divide 23 times with trachea is designated as generation zero (first generation) and the alveolar sacs as 23rd generation (last generation) (Figure 1.2.). The conducting zone comprises of first 16 generations (from trachea to terminal bronchioles) while the last 7 generations of airways which include the respiratory bronchioles, the alveolar ducts and alveolar sacs make up the respiratory zone, where the gas exchange takes place.

Figure 1.2. Weibel's lung model (tracheobronchial tree). Z = airway generation; BR = bronchus; BL = bronchiole; TBL = terminal bronchiole; RBL = respiratory bronchiole; AD = alveolar duct; AS = alveolar sac. Reproduced with permission from Ref. [5]. Copyright 1963 Springer Berlin Heidelberg

1.2. Respiratory diseases

Due to the fact that the human respiratory system is made up of many different and complicated tissues and organs, therefore, there are a number of ways and probabilities in which things can go wrong and lead to the development of respiratory diseases. Respiratory diseases range from the mild and self-limiting ones such as the common cold, to the more serious or life-threatening ones such as asthma [6, 7], chronic obstructive pulmonary disease (COPD) [6, 7], tuberculosis (TB) [7, 8], bronchitis [9], bronchiolitis [10], bacterial pneumonia [7, 11], pulmonary hypertension [12] and lung cancer [7, 13].

Respiratory diseases are affecting hundreds of millions of people around the world [14], afflicting the world population with an immense socio-economic burden. The “Big Five” of respiratory diseases: COPD, TB, acute respiratory infections (ARIs), asthma and lung cancers, account for 20% of mortality worldwide [7]. According to the World Health Organization (WHO), four of the global top ten causes of death are from respiratory disease categories [15]. These fatal respiratory diseases are lower respiratory tract infection (mostly pneumonia), COPD, TB and lung cancer.

The data from the World Lung Foundation’s first Acute Respiratory Infections Atlas shows that respiratory infections are responsible for more than 4 million deaths annually and the top killer in low- and middle-income countries [16]. Very likely, the “four million” death toll may be vastly underestimated where the real figure could be much higher due to the poor surveillance data in many developing countries. Pneumonia, which is one of the most common life-threatening respiratory infections, has been identified as the leading global killer of children less than 5 years of age. It was estimated that every 20 seconds, a child dies from pneumonia, accounting for more than 1.5 million young lives lost annually [17].

COPD is a worldwide epidemic affecting more than 200 million people with over 3 million deaths annually, making COPD as the third leading cause of death in the world [15]. It is projected that the total deaths from COPD will increase by more than 30% in the next 10 years if without the effective interventions on the underlying risk factors, particularly on tobacco use and second-hand smoke exposure [18].

Lung cancer was the most commonly diagnosed cancer in the world, contributing 13% of the total cancer diagnoses in 2012 [19]. Lung cancer also (along with trachea and bronchus cancer) ranked as the fifth leading cause of death in the world, claiming 1.6 million (2.9%) lives in 2012 [15]. A peak in lung cancer incidence is still expected unless there is further control in the prevalence of tobacco use over the next decade.

In 2012, there were about 8.6 million people fell ill with TB worldwide with an estimated 1.3 million died from the disease [18]. Geographically, the burden of TB is not evenly spread in the population across the world, but is rather concentrated in resource-limited or resource-poor countries in Asia (59%) and Africa (26%) [20]. Despite TB global mortality rate has dropped by 45% between 1990 and 2012 through the use of Directly Observed Therapy Short-course (DOTS) and the Stop TB Strategy recommended by WHO [18], the global TB-control efforts have been threatened by the emergence of multidrug-resistant TB (MRD-TB). As of 2012, cases of MDR-TB hit an all-time high worldwide, amounting approximately 500,000 new cases of MDR-TB [21].

Other common respiratory disease such as asthma, like most of the other types of respiratory diseases, is chronic in nature. This condition has emerged as a major public health problem over the past 20 years and is now afflicting about 235 million people around the world [22] and causing about 180,000 deaths worldwide each year [23]. The prevalence of asthma has increased over the last fifty years across all age, sex and racial groups with a global prevalence ranging from 1 to 18% in different countries [24]. Asthma is also the most common chronic childhood disease and always the number one chronic cause of school

absenteeism among children. In United States, an average of 1 out of every 10 school-aged children has been reported to have asthma [25].

1.3. Inhalation therapy

Inhalation (or aerosol) therapy is simply the intake of medications through nose or mouth to the lung as an aerosol for the therapeutic purposes either by natural or assisted respiration [26]. The history of inhalation therapy can be traced back to more than 3,500 years ago, where the inhalation of the vapour of black henbane was first recorded in Ancient Egyptian times (1,554 BC) for relieving the oral, pharyngeal and chest troubles [27]. Thereafter, many ancient civilizations, such as the Babylonians, Indians, Chinese, Romans and Greeks, have depicted the empirical inhalation of vapours, smoke, anaesthesia, aromatherapy and fumigation from burning the dried plants for both recreational and therapeutic purposes. There are many advantages to administer medications to the lung as an aerosol via the inhaled route over systemic administration (Table 1.1.) for treatment of respiratory diseases. Many of advantages are closely related to the main benefit of delivering high local (drug) concentrations directly to the diseased site/organ. For this reason, high therapeutic index is readily achievable together with an immediate absorption and onset of action, enabling a locally acting drug to act rapidly with minimum unwanted systemic side-effects. Typically, the adverse effects associated with inhaled medications are potentially less severe and less frequent as only a fraction of the systemic dose will be required for achieving a similar or superior therapeutic effect. For example, 100-200 µg of inhaled salbutamol (by metered-dose inhaler, MDI) is therapeutically equivalent to 2-4 mg of oral salbutamol

[28]. When a drug aerosol is delivered directly to lung, it can bypass the first-pass metabolism (destruction of drugs by liver enzymes) and avoid gastrointestinal (GI) upset, permitting more drugs to be cost-effectively and predictably bioavailable than if it were administered orally. As compared to injectable (subcutaneous or intravenous) dosage form, aerosol therapy provides a painless, non-invasive and needle-free mode of drugs delivery which greatly improves patient compliance.

Table 1.1. Advantages of inhalation drug delivery over systemic administration for treating respiratory diseases

Advantages
<ul style="list-style-type: none"> • Deliver high local (drug) concentration directly to the diseased site/organ • High therapeutic index • Immediate onset of action • A fraction of systemic dose for achieving similar or superior therapeutic effect • Less severe and less frequent of systemic adverse effects • Bypass first-pass metabolism and avoid GI upset as compared to oral administration (enhancing bioavailability) • As a painless, non-invasive and needle-free mode of drug delivery as compared to injectable form dosage (improving patient compliance)

However, it is also worthwhile to list some of the disadvantages of inhalation therapy. For example, the lack of knowledge of correct and optimal use of the inhalation devices by the patients can affect the lung deposition and dose reproducibility, therefore decrease the effectiveness of the treatment [29]. Occasionally, inhalation of high concentration of drug aerosol may cause coughing, throat irritation, hoarseness and bronchospasm in some patients, with coughing probably being the most common local side effect [30].

1.4. Advantages of dry powder inhaler (DPI)

The local and direct delivery of drug aerosols to the lung can be achieved through the inhaler devices such as nebulisers, pressurised metered dose inhalers (pMDIs) and dry powder inhalers (DPIs) [31-33]. Each type of the inhaler device is associated with its own advantages and disadvantages for various patient populations and clinical settings. The appropriate choice of inhaler device for a particular drug formulation is extremely important for successful therapy. In the optimal setting, the appropriate choice and correct use of inhaler device would have shown effectiveness both in term of the clinical benefit (adequate lung delivery) and ease of use, which in turn can improve patient's adherence with treatment and indeed long-term management and control of the respiratory diseases [34].

Nebulizers (which use a jet flow of driving gas or ultrasound to convert solutions or suspensions into small aerosol droplets) have been one of the most common means for delivering medication to lung since its first development on 1860s [35]. Typically, nebulizers can administer high doses of medication to patients with minimum patient coordination required. This advantage makes them often the favourite choice of infants, young children or elderly. However, nebulisers are often regarded as hospital or home-setting devices (due to the bulkiness) [36] with delivering fine particle fractions (FPF) of only around 10% or less to the patient [37, 38]. Hence, a longer time is required to achieve the therapeutic dose which inadvertently affects patient compliance. Furthermore, when co-nebulising two or more drug solutions together, there is the risk of undesired precipitation in solution [39]. Due to the portability and the cost-saving [40], combination medications may be found more suitable to be formulated into pMDIs than liquid nebulizers. However, pMDIs are relatively

complicated to use as good coordination between activation of the inhaler device and taking a breath is often essential to ensure efficient drug deposition in lung [40]. Poor coordination between actuation and inhalation (either the patient inhales too rapidly or too slowly) can affect the delivery of medication to the lower airways, resulting only a small fraction of medication reaching the disease site. Besides, the propellant used in pMDIs (e.g. hydrofluoroalkane, HFA) is green house gas that may contribute to global warming. This propellant is also typically cold and can generate a cold sensation in patients' pharynx, causing them to have a cough reaction and stop inhaling (so-called the cold freon effect) [41]. A DPI formulation is hence favoured over the other aerosol delivery modes (i.e. nebuliser or pMDI) in delivering combination medication mainly due to the improved formulation stability associated with the powdered drug (as compared to the solution or suspension in the pMDI and nebuliser) and the avoidance of undesired precipitation in solutions (e.g. in the nebuliser). Other advantages of DPIs include improved delivery efficiency, portability, ease-of-use (require little or no coordination of actuation and inhalation) and free of cold freon effect [37, 42-44]. Furthermore, the possibility of high drug dosing flexibility of DPIs enable a range of doses from 6 µg to 20 mg (or more) to be delivered per shot [45]. This high drug dose carrying capacity is very useful and important especially for antibiotic combination powders which generally require a relatively high lung dose (i.e. 20-100 mg) [46].

1.5. Powder production methods for DPIs

Drug particles with aerodynamic diameters of 1-5 µm are optimal for the deposition in the transitional and respiratory zones of the lung and hence are

found to be most useful for inhaled drug delivery [47]. Several micronization techniques such as milling, spray-drying, spray freeze-drying and supercritical fluids are available to prepare particles with respirable size range (i.e. 1-5 μm) [48]. Each of the techniques will be discussed in more detail below.

1.5.1. Milling

Milling is the traditional method of drug powder micronization [49]. It is regarded as a “top-down” approach in the production of fine drug powders as it involves the use of mechanical forces to physically break down the large coarse drug particles into finer particles with reduced size [50]. Very often, the term milling, grinding, comminution and pulverization are used interchangeably. Various mills like ball mills, pin mills and jet mills (fluid energy) have been well-established and widely used for manufacturing dry powders for inhalation [51]. Figure 1.3. shows the basic designs of three mills that commonly used to create micron-sized particles.

Figure 1.3. Micronization. Cross-sections of three mills commonly used to create micron-size particles. A: Ball mill. B: Pin mill. C: Jet mill. Reproduced with permission from Ref. [51]. Copyright 2005 Daedalus Enterprises Inc.

Both the ball milling and pin milling use mechanical impact (i.e. particle-particle and particle-solid collisions) to grind powder. In ball milling, drug powder is loaded into a drum and then grounded by the grinding media such as ceramic balls or stainless steel balls as the drum rotates around a horizontal axis [52]. The rolling balls grind the coarse particles between each other during the rotation of drum. In pin mill, a series of concentrically mounted pin is attached to the rotating plate of a pin mill in order to achieve high energy impact between the mill and the drug particles. Centrifuge force is applied to smash coarse particles by impact [51, 53]. Unlike ball milling and pin milling where they use mechanical component to grind particles, jet milling uses high pressure air to generate high particle velocity and hence high energy impact between particles. Coarse particles are fractured into fine fraction upon contact with gas stream due to inter-particle collision and attrition [53].

Although milling is a cost-effective way to generate micron-sized particles, it is used with limited success [49, 54]. A major disadvantage of milling is that there is only limited control over the particle characteristics such as size, shape and morphology [49]. The excess energy supply during milling could also lead to the amorphous phase formation at the surface of fractured crystals. Therefore, the powder would tend to recrystallize from the partially amorphous state during manufacturing and storage, causing potential chemical and physical instability problem [55, 56]. Besides, the micronized particles produced by milling process typically carry high amounts of charge and possess high surface free energies [57, 58] as a result of friction and contact with mill surface (i.e. triboelectrification process), making them highly cohesive and prone to agglomeration and aggregation. These undesirable characteristics have hence compromised the aerosolization performance of drug powders [57].

Furthermore, many materials especially thermo-labile biopharmaceuticals like peptides and proteins, and brittle molecules like porous/hollow particles are not able to withstand the abrasion and shearing during milling process [57, 59, 60].

1.5.2. Spray drying

Spray drying is a well-established method commonly used in the pharmaceutical industry for producing a dry powder from a liquid phase [61]. Among the powder production methods, spray drying has been used extensively for the preparation of drug particles intended for inhalation drug delivery [62, 63]. It offers the advantage of drying and particle formation in a single-step continuous and scalable process with particle engineering possibilities [64]. Furthermore, various particle properties such as particle size, bulk density and flow properties can easily be tuned via simple manipulation of the process parameters or spray dryer configuration. Therefore, spray drying is potentially a versatile and commercially-viable technique for formulating inhalable drugs. However, the major drawback of spray drying is that most materials undergo amorphization, which could affect the stability of powder [65]. These stability issues could be ameliorated by formulation measures, such as co-spray drying the active pharmaceutical ingredients (APIs) with an excipient into a stable amorphous solid dispersion [66].

The typical spray drying process encompasses four fundamental steps: (a) atomization of feed into a spray, (b) spray-air contact, (c) drying of spray, and (d) separation of dried product from the drying air [64]. A liquid feedstock is atomized into a spray of fine droplets by centrifugal energy (using rotary atomizer), pressure energy (using pressure nozzle) or kinetic energy (using two-fluid nozzle) and then brought into contact with the hot drying gas (e.g. air or

inert gases) at sufficient temperature for the moisture evaporation to take place. In the conventional spray dryer chamber, turbulent flow is used to promote more efficient drying. However, the dispersed droplets are also subjected to different local air temperature and humidity conditions as a result of non-laminar gas flow through the system [67], resulting to differences in drying degree and hence, the potential non-homogeneity in particle size and morphology [68]. As the moisture evaporates from the droplets, the solid product is formed, and the powder is readily recovered from the drying gas. The cyclone is currently the most commonly applied device for separation and collection of particles. Separation is brought about via the setup of centrifugal forces arising from the highly rotated air stream, which propels the particles towards the wall of device [67]. Figure 1.4. shows a photograph and a schematic diagram of the conventional spray dryer (i.e. Buchi[®] Mini Spray Dryer B-290).

Figure 1.4. (Left): Photograph and (Right): A schematic diagram of the conventional spray dryer. Adapted and reproduced with permission from Ref. [69]. Copyright 2011 BÜCHI Labortechnik AG

The field of spray drying is constantly evolving. Since the first development of spray drying technology in the early 1870 [67], there have been numerous efforts undertaken to refine the equipment and technology to ensure relevance and attractiveness to the food and pharmaceutical industry. The recent advance in spray drying technology would be the development of the Buchi® Nano Spray Dryer B-90 which has incorporated three innovative technologies in spray head, heating system and particle collector [68]. The Nano Spray Dryer B-90 is the fourth and newest generation of the laboratory-scale spray dryers developed by Buchi®, following on the success of the previous three generations (i.e., Mini Spray Dryers B-190, B-191 and B-290) since the 1970s [70]. This laboratory scale spray dryer is able to generate narrow-distributed

particles in the size range of 300 nm to 5 μm for milligram sample quantities at high yields [63, 70]. A narrow size distribution will not only increase the efficiency of delivery to specific region of the lung, but will also minimize oral deposition, making this technology highly suited for inhalation drug delivery [68]. Figure 1.5. shows a photograph and a schematic diagram of the Nano Spray Dryer B-90.

Figure 1.5. (Left): Photograph and (Right): A schematic diagram of the Nano Spray Dryer B-90. Reproduced with permission from Refs. [70, 71]. Copyright 2009 BÜCHI Labortechnik AG & 2011 Elsevier

The new Nano Spray Dryer B-90 utilizes a vibrating mesh technology for fine droplets generation. Basically, the piezoelectric crystal driven spray head is incorporated with a small spray cap that contains a thin perforated membrane (spray mesh) having an array of precise micron-sized holes (i.e. spray meshes of 4 μm , 5.5 μm or 7 μm hole size). When the piezoelectric actuator is driven at an ultrasonic frequency (i.e. 60 kHz), the mesh will vibrate upwards and downwards, injecting millions of precisely sized droplets from the holes and

generating the aerosols. Figure 1.6. illustrates the functional principle of mesh vibration occurring at the piezoelectric driven spray head of the Nano Spray Dryer B-90.

Figure 1.6. The functional principle of mesh vibration occurring at the piezoelectric driven spray head of the Nano Spray Dryer B-90. Reproduced with permission from Refs. [70, 71]. Copyright 2009 BÜCHI Labortechnik AG & 2011 Elsevier

The heating system of the Nano Spray Dryer B-90 operates on a laminar flow principle, whereby the laminar flow is generated by air passing through a compact porous metal foam that is conducive for optimal energy input and has short heating-up rates. With laminar flow, gentle heating is achievable, thus making the system extremely ideal for heat-sensitive biopharmaceutical products. Furthermore, the vertical configuration of the spray dryer facilitates direct and straight-down collection of the particles into the collector, which helps to minimize particle adherence to the side walls of the glass chamber, and hence allowing for much higher collection yields.

In contrast to the common cyclone technology where particles smaller than 2 μm are typically not captured [72], particle separation in the Nano Spray Dryer B-90 involves the use of the electrostatic precipitator whereby the collection

mechanism is independent of particle mass. Collection of fine particles with high efficiency is achieved with the novel electrostatic particle collector consisting of a grounded star electrode (cathode) and cylindrical particle collecting electrode (anode). The presence of a high voltage around the particle collector creates an electrostatic field that accelerates the deposition of negatively charged particle onto the inner wall of particle collecting electrode. This is followed by a discharging process. Figure 1.7. illustrates the functional principle of an electrostatic particle collector in the Nano Spray Dryer B-90.

Figure 1.7. The functional principle of an electrostatic particle collector in the Nano Spray Dryer B-90. Reproduced with permission from Refs. [70, 71]. Copyright 2009 BÜCHI Labortechnik AG and 2011 Elsevier

1.5.3. Spray freeze drying

Spray freeze-drying was first introduced in 1994 [49]. It is an advanced two-step particle engineering technique that combines spray-drying and freeze-drying processing steps. It involves freezing of the spray droplets in a cryogenic liquid such as nitrogen, oxygen or argon, followed by lyophilization of the

frozen droplets to remove water via sublimation, then leaving behind a low density, amorphous and porous particles with large surface areas (Figure 1.8.) [48, 73, 74].

Figure 1.8. Schematic of the spray freeze drying process. Reproduced with permission from Ref. [48]. Copyright 2015 Bentham Science

This method has been reported to preserve integrity of highly thermo-labile proteins and peptides [75, 76] and produce light and porous particles with enhanced aerosol performance [73, 77-79]. Biotherapeutics such as insulin, trypsinogen, plasmid DNA and influenza vaccine have been successfully formulated into inhalable dry powders using spray-freeze drying [80-83].

However, spray-freeze drying is not as commonly used as spray drying due to its high cost, long processing time, exposure to the shear and freezing stresses, and difficulty in scaling up [49, 57]. Combining a spray dryer with a lyophilizer makes the technique expensive. The cost can be easily 30-50 times more expensive than spray drying [84]. Furthermore, it is a time consuming process where a full spray-freeze drying process may take up to three days [49]. The stresses associated with shear, freezing and drying that experienced in spray-freezing drying have also limited its success use in producing protein/peptide particles. Irreversible protein damages like structural denaturation and

aggregation have been reported [85, 86]. Moreover, the inconvenience of handling cryogenic fluids that typically have low boiling points below -150°C has made the scaling up of spray-freeze drying a challenging process [87].

1.5.4. Supercritical Fluid Technology

Supercritical fluid (SCF) technology is a relatively new powder production method that is still under development. It has been an attractive method for producing dry powder due to its solvent-free nature. Carbon dioxide (CO₂) is the most widely used supercritical fluid as it is chemically inert, non-toxic, non-flammable, highly pure, inexpensive and has a low critical point (critical temperature = 31°C; critical pressure = 74 bar) [88]. In particular, its low critical temperature makes it highly suitable for thermo-labile materials. With this technique, production of crystalline micron-sized powder with defined morphology and uniform particle size distribution could be obtained in a single-step operation [88, 89].

Generally, there are two main routes for particle formation with supercritical fluid, which are precipitation from supercritical fluid solutions (e.g. Rapid Expansion of Supercritical Solution, RESS) and precipitation using supercritical fluids as anti-solvents (e.g. Supercritical Antisolvent, SAS). In RESS, supercritical CO₂ is used as a solvent to dissolve drug solute and then the drug particles are precipitated by rapid expansion of the supercritical fluid (Figure 1.9.) [90-92]. However, the application of RESS process in particle formation is limited by the poor solubility of most APIs in supercritical CO₂ [93].

Figure 1.9. Schematic of the supercritical fluid process, (A) using SCF as the solvent and (B) using SCF as the anti-solvent. Reproduced with permission from Ref. [92]. Copyright 2011 Elsevier

To overcome the limitation of RESS, other supercritical process such as SAS has been developed where the supercritical CO_2 is employed as a non-solvent. In SAS, the drug substance is dissolved in a solvent that is miscible with supercritical CO_2 . The solubilisation power of the solvent decreases as the CO_2 is incorporated, resulting in precipitation of drug particles (Figure 1.9.) [88, 92]. There are also other variants to the technology such as solution enhanced dispersion by supercritical fluids (SEDS) [94], aerosol solvent extraction system (ASES) [95], polymerization-induced phase separation (PIPS) [96], supercritical solvent impregnation (SSI) [97] and supercritical assisted atomisation (SAA) [98], but all of them function with same principle of precipitation of the drugs via solvent extraction with supercritical fluid CO_2 [57]. Despite the exciting potentials, this technology is still in its infancy and has yet to be harnessed in marketed DPI products. The major disadvantages of this technology are the high machine expenditure and difficulty in scaling-up [88]. The elevated pressure (up to hundreds of bars) required to maintain solvent in supercritical state (i.e. safety issue) [99] and the limited availability of solvents have further limited its broad application [88].

1.5.5. The choice of powder production methods for DPIs

In this thesis, spray drying was selected as the powder production method for preparing the inhaled combination powders. To generate inhaled particles, bottom-up processing approach (e.g. spray drying, spray-freeze drying and supercritical fluid technology) is preferred over top-down approach (e.g. milling) as it offers greater control of particle properties, including particle size and distribution, morphology, porosity, density and surface energy [100]. In addition, milling requires high energy or pressure to achieve optimal comminution, which may lead to contamination with impurities when a milling medium is used [101]. The high surface energies of milled particles could further lead to aggregation, resulting in poor powder aerosolization performance [57, 58]. Among the bottom-up processing approaches, spray drying has been our choice of method due to its popularity in manufacturing respirable particles [102, 103]. It is capable of processing a wide variety of materials in a rapid, continuous, cost-effective, reproducible and scalable way [61]. Other bottom-up processing methods, like spray-freeze drying and supercritical fluid technology, are either associated with lengthy process time [87] or safety risk (i.e. high pressures) [99]. Moreover, they are high-cost equipments and mostly used on a small scale, particularly for research purposes only [87, 88]. Although the amorphization process may compromise the stability of the spray-dried powder, various types of excipient may be added to the formulation to enhance the stability of amorphous spray-dried powders, while retaining the benefits of amorphous particles in improving bioavailability and dissolution rate [104]. The powder production method chosen for this thesis was based on the economics of the method and its applicability to the pharmaceutical industry.

1.6. Combination therapy

Combination therapy (namely, a cocktail of drugs) can be achieved through the use of two or more APIs with complementary modes of action either administered separately or in a single-dosage formulation [105]. As compared to the former, the latter (also known as fixed-dose combination) is relatively more popular in the clinical practice and has been flourishing in the pharmaceutical market owing to its advantages in term of improved clinical effectiveness, enhanced patient adherence and reduced administrative cost [106]. In the context of this thesis, we shall regard “combination therapy” as “combination therapy with fixed-dose combination”.

Unlike monotherapy, the use of multiple drugs with different modes of action in combination therapy allows them to act independently from each other at the different action sites of disease, leading to the improved treatment efficacy and, possibly, overcoming the mechanisms of resistance. Table 1.2. shows the advantages of combination therapy. The most major advantage of combination therapy is the potentiation (or enhancement) effect of one drug by another so that the combined effect is greater than the sum of the effects of each one alone [107, 108]. In combination antibiotic therapy, this combined effect is termed as synergistic effect, where the combined effect of two antimicrobial agents together being greater than the sum of their individual activities [109]. This condition has not only increased the efficacy of the therapeutic effect, but also has decreased the total drug doses and/or duration of treatment, and thereby minimizing the adverse effects of each individual drug [107, 108]. When a lower drug dosage is required for achieving enhanced or maintaining same therapeutic efficacy, development and emergence of drug resistance could potentially be prevented or delayed also [107, 108]. In addition to the aspect of

improved clinical effectiveness, combination therapy has the advantage of improving patient compliance by offering simpler dosage schedules with fewer medications a patient has to take or even once-daily dosing [106]. With such reduced complexity drug regimens, the likelihood of patient adherence to treatment can hence be promoted and therefore improving the treatment outcome indirectly. In term of economic evaluation, combination therapy may be considered as more cost-effective than the medications administered separately. Reduction in manufacturing cost (upon simplified packaging) and administration cost (due to fewer prescriptions and dispensing fees) [106] can greatly lessen the overall health care financial burden. With all these benefits in view, combination therapy has been applied widely by clinicians in practice to treat various human diseases, such as diabetes [110, 111], hypertension [112, 113], cardiovascular diseases [114, 115], cancer [116, 117], malaria [118, 119], HIV/AIDS [120, 121] as well as respiratory diseases [122, 123] (which is the main focus of this thesis).

Table 1.2. Advantages of combination therapy

Advantages
<ul style="list-style-type: none"> • enhance therapeutic efficacy (potentiation effect of one drug over another) • provide synergistic effect (antibiotic) • reduce duration and total drug dose, minimizing toxicity (side effects) • delay emergence or prevent development of drug resistance • improve patient compliance and adherence • reduce manufacturing cost (simplified packaging) and administration cost (fewer prescription and dispensing)

1.6.1. Inhaled combination formulations for asthma and COPD

The clinical benefit of combination therapy for asthma and COPD has been well established and its growing popularity is reflected in the commercially available and clinical development of a large number of combination products

worldwide (Table 1.3.) [124]. Although the majority of the commercial combination products in current market are in pMDI form, DPI is expected to be used increasingly in the future as the most common inhaled dosage form for delivering combination products due to its advantages as discussed in Section 1.4.

For the past two decades, the use of an inhaled corticosteroid (ICS), combined with an inhaled long-acting β_2 -agonist (LABA) has been well-recognized as the gold standard therapy for asthma that is not controlled on low doses of ICS monotherapy [21, 125, 126]. Current Global Initiative for Chronic Lung Disease (GOLD) guidelines recommend that a combined regimen of ICS and LABA to be used in COPD patients with GOLD stage III (severe, $FEV_1 = 30\%-50\%$) and IV (very severe, $FEV_1 < 30\%$) with a history of repeated exacerbations (e.g. three in three years) or chronic symptoms [127]. The scientific rationale for combining an inhaled ICS with an inhaled LABA arises from the strong complementary effects on the different physiopathological components of asthma and COPD (such as inflammation and bronchial obstruction) that neither drug class is able to achieve by its own and also the positive interaction between ICS and LABA [126, 128, 129]. Inhaled ICSs suppress airway inflammation at relatively low doses, whereas inhaled LABAs relax airway smooth muscle, inhibit mast cell mediator release, prevent plasma exudation and reduce sensory nerve activation [126]. Table 1.3. shows the examples of inhaled ICS-LABA combination products that are currently commercially available and under clinical trial. Among them, GlaxoSmithKline's Seretide[®] and Astra Zeneca's Symbicort[®] are the leading combination products which have revolutionized the treatment of asthma and COPD.

There is a widespread use of short-acting bronchodilator (β_2 -agonists (SABAs) and anti-muscarinic antagonists (SAMAs)) for the management of moderate-to-severe COPD, either as individual agent or in combination [130]. The scientific rationale for SABA/ SAMA combination therapy lies on the maximum bronchodilation that achieved via dual bronchodilation with SABA and SAMA and the co-existences of complimentary sites of actions, which SAMA effect concentrates in the proximal bronchial smooth muscle (more central airways) and SABA effect concentrates in the distal bronchioles (smaller peripheral airways). A combination of the two classes is hence able to target both sites of action [53, 54]. A fixed-dose combination of the short-acting agents ipratropium and albuterol (e.g. Combivent[®]) and fenoterol and ipratropium (e.g. Berodual[®]) have been reported to provide superior therapeutic outcomes over monotherapy with either agent alone [131-133].

The combination of a long-acting β_2 -agonist (LABA) with a long-acting anti-muscarinic antagonist (LAMA) has been recommended as the second-line therapy in the management of COPD where sustained bronchodilation is required [127, 134]. The dual parallel bronchodilating modes of actions of LABAs and LAMAs provide better bronchodilation than the any individual agents and hence offer them an attractive combination therapy for COPD [135, 136]. Duova[®] (Cipla) is a well-established marketed fixed-dose combination of LABA/LAMA (i.e. formoterol and tiotropium) available for patients with COPD. Generally, LAMAs prevent smooth-muscle contraction through prolonging the blockade of muscarinic (M3) receptors which are the mediators for bronchoconstriction and mucus secretion [137, 138], whereas, LABAs acts to relax airway smooth muscle by stimulating β_2 -adrenergic receptors and providing functional antagonism to bronchoconstriction [139]. Given that the

importance of LABA/LAMA combination therapy, more new combinations of LABA/LAMA are currently under development (Table 1.3.), such as vilanterol/umeclidinium bromide, formoterol/aclidinium and olodaterol/tiopropium [124].

As COPD progress, triple inhaled therapy of a LABA, LAMA and ICS has also been recommended for patients with concomitant asthma and persistent exacerbations [109, 140]. As a general rule of thumb, monotherapy is always attempted first and then intensified to dual therapy and ended with triple therapy if necessary [125]. Clinical evidence to date indicates that patients with advanced COPD can be beneficial from the triple inhaled therapy of a LABA, LAMA and ICS, mainly due to their complementary modes of action in maintaining airway functions [124, 141]. Such evidence has then served as the basis for the development of the triple combination product in market and under clinical investigation (Table 1.3.). Although Triohale (Cipla) is the only marketed triple combination product now, it is believed that more triple combination products will be under development in near future and become one of the important clinical practice for the management of COPD.

Table 1.3. Examples of inhaled combination formulations (both marketed and in clinical development) for the treatment of asthma and COPD

Active Ingredients			Brand names (Region)	Product Type	Manufacturer	Status
(a) ICS + LABA						
<u>ICS</u>	<u>LABA</u>					
Fluticasone propionate	Salmeterol xinafoate		Advair® (US & Canada)	pMDI & DPI	GlaxoSmithKline	Market
			Seretide® (EU, Worldwide)	pMDI & DPI	GlaxoSmithKline	Market
			Viani® (Germany)	pMDI & DPI	GlaxoSmithKline	Market
			Adxair® (Japan)	pMDI & DPI	GlaxoSmithKline	Market
			Foxair® (South Africa)	pMDI & DPI	GlaxoSmithKline	Market
			Serflo® (Worldwide)	pMDI & DPI	Cipla	Market
Budesonide	Formeterol fumarate		Symbicort® (USA)	pMDI	Astra Zeneca	Market
			Symbicort® (EU, Worldwide)	DPI	Astra Zeneca	Market
			Foracort® (Worldwide)	pMDI & DPI	Cipla	Market
Beclomethasone dipropionate	Formeterol fumarate		Foster® (EU)	pMPI	Chiesi	Market
			Foster®NEXhaler® (EU)	DPI	Chiesi	Market
			Formodual® (EU)	pMDI	Chiesi	Market
			Innovair® (EU)	pMDI	Chiesi	Market
Mometasone furoate	Formeterol fumarate		Dulera® (US)	pMDI	Merck & Co.	Market
			Zenhale® (Canada)	pMDI	Merck & Co.	Market
Fluticasone	Formeterol fumarate		Flutiform® (EU, Japan)	pMDI	SkyPharma	Market
Fluticasone	Vilanterol trifenate		Relvar Ellipta® (EU)	DPI	GlaxoSmithKline	Market
			Breo Ellipta® (US)	DPI	GlaxoSmithKline	Market
Mometasone furoate	Indacaterol		QMF 149	DPI	Norvatis	Phase III
Budesonide	Carmoterol		DHF-5188	pMDI	Chiesi	Phase II
(b) SABA + SAMA						
<u>SABA</u>	<u>SAMA</u>					
Albuterol sulfate	Ipratropium bromide		Combivent® (US, EU, Worldwide)	nebuliser & pMDI	Boehringer Ingelheim	Market
			Duoneb® (US)	nebuliser	Mylan	Market
			Ipramol® (EU)	nebuliser & DPI	TEVA	Market
			Duolin® (Worldwide)	Nebuliser, pMDI & DPI	Cipla	Market
Fenoterol	Ipratropium bromide		Duovent®	Nebulizer & pMDI	Boehringer Ingelheim	Market
			Berodual®	Nebulizer & pMDI?	Boehringer Ingelheim	Market
			Bronchodual®	Nebulizer & pMDI?	Boehringer Ingelheim	Market
			Atrovent®	Nebulizer & pMDI?	Boehringer Ingelheim	Market
(c) LABA + LAMA						
<u>LABA</u>	<u>LAMA</u>					
formoterol	tiotropium		Duova®	pMDI & DPI	Cipla	Market
Indacaterol	Glycopyrrolate		Ulibro® Breezhaler®	DPI	Novartis	Market
Vilanterol	umeclidinium bromide		OVA 149			
			Anoro Ellipta	DPI	GlaxoSmithKline & Theravance, Inc.	Approved
Formoterol	acilidinium		Duakir	DPI	Amirall & Forest Laboratories	Phase III
Olodaterol	tiotropium		Striverdi® Respimat®	Respimat®	Boehringer Ingelheim	Phase III
formoterol	Glycopyrrolate		PT003	pMDI	Pearl Therapeutics	Phase III
(d) ICS + LABA + LAMA						
<u>ICS</u>	<u>LABA</u>	<u>LAMA</u>				
Ciclesonide	formoterol	tiotropium	Triohale	pMDI	Cipla	Market
budesonide	formoterol	glycopyrronium	PT010	pMDI	Pearl Therapeutics	Phase III
Fluticasone propionate	Vilanterol	umeclidinium bromide		DPI	GlaxoSmithKline & Theravance, Inc.	Phase III

Data modified from [142]

1.6.2. Inhaled combination formulations for respiratory infections

Due to the large surface area ($\sim 140 \text{ m}^2$, even larger than that of small intestine) exposed to the external environment and minimum barrier defence, the lungs become extremely vulnerable to serious infection [143]. Most of the common respiratory diseases or respiratory bacteria-linked respiratory diseases such as pneumonia, bronchiectasis, COPD and cystic fibrosis (CF) are intimately associated to airway infections. Nonetheless, the architecture of lung also offers great potential for the targeted delivery of high concentration of antimicrobial agents directly to the infection site in the lung, ultimately enhancing the bacterial kill while minimising the systemic toxicities. The use of aerosolized antimicrobial therapy in clinical practice has started as early as 1950s [144], with the initial attempts at aerosolizing neomycin and penicillin to treat pneumonias in adults [145]. Subsequently, over the next 20 years, the clinical availability of other less toxic aminoglycosides such as gentamicin and tobramycin has then further expanded the aerosol antibiotic choices for the treatment of airway infections [145-147]. There are also increasing rigorous clinical studies on a series of inhaled antimicrobial agents such as polymyxins [148, 149], fluoroquinolones [150, 151] and β -lactams [152, 153] for the treatment of airway infections since the early 1980s. However, most studies on antimicrobial aerosolization are focus on the nebulizer forms which allows the use of intravenous antimicrobial solution with little or no-reformulation [36]. As these intravenous antimicrobial solutions were not intended for inhalation, they are hence typically associated with unpleasant side effect such as airway irritation, coughing and bronchoconstriction (upon nebulisation) due to the pH issues and preservatives in the solutions [154]. Research efforts have hence shifted to focus on a faster, more efficient and stable aerosol delivery method

which uses dry powder technology. The two recently launched commercial antibiotic DPI formulations, TOBI®Podhaler™ (tobramycin powder for inhalation) and Colobreathe® (colistimethate sodium powder for inhalation) for the treatment of chronic *Pseudomonas aeruginosa* lung infection in patients with CF, have reflected the increasing importance of antibiotic DPI formulations. Parallely, there has also been a movement of antibiotic dry powder formulations towards combination antibiotic therapy for respiratory infections, especially in the academic scene.

Inhaled combination antibiotic therapy is an important treatment modality in respiratory infectious disease. It is mostly practised in clinical setting in an attempt to (i) create antibiotic synergism (synergy is defined as combined effect of two antimicrobial agents together being greater than the sum of their individual activities [109]) so as to improve pharmacotherapeutic efficacy; (ii) to minimize the risk of drug toxicity by allowing the use of lower doses than would be used in monotherapy (as a result of the synergism effect of combined regimen and the drug localization effect at desired site of action upon aerosolization); (iii) to counteract the development of resistant bacterial variants (as chances of emergence of resistance against multiple drugs are lower as compared to a single drug) and (iv) to widen antibacterial spectrum of the empirical therapy (as the infecting pathogen would have been covered by at least one of the multiple components of the regimen [155]).

The effort of combining and co-delivering two antibiotics in a single inhalable dry powder was first described by Adi et al. in 2008 [156]. Adi et al. [156] had prepared an inhalable co-spray dried antibiotic (ciprofloxacin hydrochloride and doxycycline hydrochloride) formulation with acceptable FPFs and

improved physical stability, but the formulation was found to be non-synergistic via the qualitative disk diffusion test. The result was in line with an earlier report which reported the ciprofloxacin-doxycycline pair as being suppressive (or antagonistic at low concentrations) [157]. After the initial effort by Adi et al. [156], Tsifansky et al. [158] reported the co-encapsulation of ciprofloxacin and ceftazimide in a dry powder platform containing dipalmitoylphosphatidylcholine (DPPC), albumin and lactose for intrapulmonary delivery and demonstrated the additive anti-pseudomonas activity against a tested strain of *Pseudomonas aeruginosa*. Although both studies by Adi et al. [156] and Tsifansky et al. [158] demonstrated the uses of their combination antibiotic formulations as potential DPI systems, synergism was not achieved in their formulations.

Lee et al. [159] then took one step further by developing a synergistic DPI formulation against *Pseudomonas aeruginosa* that contains ciprofloxacin hydrochloride and gatifloxacin hydrochloride in weight ratio of 1:2.5. In contrast to the previous work by Adi et al. [156] and Tsifansky et al. [158] where the combination ratios were quite arbitrarily determined, the choice of drug ratio of the combination formulation of this work was clearly addressed. The antibiotics' minimum inhibitory concentration (MIC) values were first determined experimentally via microdilution method and then used as preliminary guidance for each antibiotic's required therapeutic dose in the combination formulation (i.e. combination ratios). This binary formulation was of respirable-size (D_{50} of $1.83 \pm 0.08 \mu\text{m}$) and capable of achieving a fine particle fraction (FPF) of approximately 40% with a concomitant and uniform in vitro deposition profile across all impaction stages when dispersed at 60 L/min. Co-spray dried powder of ciprofloxacin hydrochloride and gatifloxacin

hydrochloride may therefore offer an alternative anti-pseudomonal therapy to control the airway infections associated with cystic fibrosis and pneumonia [159].

In the following year to Lee et al.'s work [159], Zhou et al. [160] had prepared an inhalable combination powder formulation consisting of colistin and rifampicin by co-spray drying in a mass ratio of 1:1 based upon the recommended dosage regimens of both antibiotics and in vitro synergy data published by Li et al. [161]. The combination formulation had a high aerosolisation performance with the emitted dose and FPF total over 90% of the recovered dose. However, the antibacterial activity of the combination powder was not tested against any multi-drug resistant (MDR) bacteria although the combination formulation aimed to treat respiratory infections caused by MDR Gram-negative bacteria.

Besides the combination of various antibiotics, several studies have demonstrated the feasibility of incorporating non-antibiotic adjuvants such as mucolytic agents in the antibiotic dry powder formulations. For example, Yan et al. [162] reported the co-delivery of ciprofloxacin (Cipro; an antibiotic) and deoxyribonuclease (DNase; a mucolytic) using an inhalable particle system consisting of DPPC, albumin and lactose in weight ratio of 3:1:1. The results showed that Cipro/DNase powder had better antibacterial activity than Cipro powder when applied to artificial sputum laden with *Pseudomonas aeruginosa*. The higher activity of the Cipro/DNase powder was ascribed to the mucolytic activity of DNase in decreasing sputum viscoelasticity and hence promoting the penetration of the dry powder into the artificial sputum and efficient diffusion of ciprofloxacin. Nevertheless, the substantial cost of DNase presents an

additional challenge to its routine use. Therefore, Yeo et al. [162] had explored the use of alternative osmotic agents such as mannitol and sodium chloride (NaCl). The study showed that cipro-mannitol (5%) particles had higher antibacterial activity than cipro-NaCl (5%) particle in *Pseudomonas*-laden artificial mucus (AM) although their (NaCl and mannitol) effect on rheological properties of AM appeared to be similar. The superior antibacterial activity of cipro-mannitol particles was likely attributed to mannitol's influence on the AM environment, namely the ability to induce the water flux and penetrate the AM, which had visualised by transmucus diffusion of Coomassie blue (CB)-mannitol (i.e. CB in CB-mannitol particles penetrated the AM faster than the dye alone or that in CB-NaCl). Mannitol has also been incorporated into the ciprofloxacin DPI formulation in Adi et al's work. [163]. It was found that when mannitol and ciprofloxacin were formulated in a co-spray dried powder in a 50:50 weight ratio, a physically stable and efficient aerosol powder could be produced with no influence on the antimicrobial activity with respect to *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Streptococcus pyogenes*.

1.7. Research motivation and scientific contribution

Inhaled antibiotics have been used clinically to treat respiratory infections since the 1950s [144] with the aim of targeting the antibiotics to the site of respiratory infections and minimizing systemic exposure and the associated toxicity risks. However, most of the early inhaled antibiotic formulations involved the use of antibiotics that were meant for parenteral administration. These formulations were typically poorly tolerated by the patients due to the added preservatives and non-physiologic chemical composition, which could

inadvertently promote bronchial irritation and bronchospasm [164]. The recent launch and commercialization of TOBI Podhaler[®] (tobramycin inhalation powder, Novartis Pharmaceuticals) and Colobreathe[®] (colistimethate sodium inhalation powder, Actavis UK Ltd) have marked a major therapeutic advance with the focus on dry-powder development for fast, convenient and stable delivery of inhaled antibiotics. To date, there has been no known antibiotic-antibiotic or antibiotic-(non-antibiotic) combination DPIs available commercially. Antibiotic combinations are commonly engineered in medicine to broaden the antimicrobial spectrum, in an attempt to combat the spread of antibiotic-resistant bacterial pathogen and also to generate synergistic effects [106, 157]. Non-antibiotic drugs in the combination of antibiotics with non-antibiotic drugs could potentially augment the activity of the antibiotic/s to bring about a more favourable outcome to the patient. This research work aims to develop several combinatorial antibiotic DPI formulations containing non-antibiotic adjuvants such as a corticosteroid (Chapter 3) and a mucoactive agent (Chapter 4) for respiratory infections complicated with airway inflammation (Chapter 3) and with mucus hypersecretion (Chapter 4), respectively. Novel combinatorial antibiotic DPI formulations containing triple antibiotics from different antibiotic classes have also been designed for rotational use against respiratory “superbugs” (Chapter 5). Antibiotic combinations must be rationally chosen to exploit potential synergism and avoid undesired antagonism in their anti-bacterial activities.

Due to the multicomponent nature and molecular complexity of inflammatory airway disorders, such as COPD and asthma, inhaled combination therapies with complementary modes of action offers effective treatment for airway inflammation. Inhaled corticosteroids (either alone or in combination with other

drugs) often form the backbone of anti-inflammatory therapy. However, there are always some potentially adverse reactions from inhaled corticosteroids. As compared to corticosteroids, natural agents that induce very few side effects may be a beneficial alternative to combat airway inflammation in a gentler and safer way, and may also be a useful alternative for treating mainstream corticosteroids-resistant inflammation. A novel inhalable non-steroidal natural dry powder combination formulation has been developed and studied for anti-inflammatory therapy (Chapter 6). This inhalable nutraceutical-based combination powder has the potential to provide an inexpensive and safer treatment alternative to the conventional inhaled corticosteroids.

There are very limited examples of marketed dry powder inhalation combination products for the treatment of respiratory diseases or disorders. Seretide[®] (salmeterol xinafoate/fluticasone propionate, GlaxoSmithKline) and Symbicort[®] (budesonide/formoterol, AstraZeneca) are the two most successful marketed combination products for asthma therapy. These marketed combination dry powder formulations are typically prepared by geometric blending of both the micronized active pharmaceutical ingredients (APIs) together with coarse lactose as a carrier [165]. However, there is little control in powder blend uniformity (i.e. the API distribution or homogeneity in the “final” blend or mix). Hence, much effort has been taken to explore the alternative ways for preparing combination dry powder formulations with good drug uniformity. Spray-drying, being a fast and simple method of liquid to solid conversion with the ease of scale-up is used in our research work to demonstrate the concept of co-formulating multiple APIs into a single dry powder dose with good drug uniformity. Unlike geometric blending where the particles of individual API are physically mixed, spray-drying allows the

preparation of individual single particulate with two or more APIs in pre-determined ratios for uniform and concomitant drug delivery. Particle properties, such as particle size, particle shape, particle density and surface roughness, of the combination powder can be easily manipulated by varying the spray-drying process parameters to obtain an ideal DPI formulation with good powder de-aggregation and dispersion, deep lung deposition with increased fine particle fraction (FPF, $\leq 5 \mu\text{m}$) and decreased device retention.

The research work in this thesis is particularly relevant and timely in an era of heightened occurrence of respiratory system infections and disorders. The novel combination dry powder formulations of this thesis provide new and valuable insights to the community of clinicians and researchers especially involved in the work of finding cures or adjuvant therapies for respiratory bacterial infection and airway inflammation.

Figure 1.10. The preparation and working procedures of the dry powder combination formulations for the treatment of respiratory infections and disorders. Diagram not drawn to scale

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Chapter 2

Materials and Methods

2.1. Materials

Ciprofloxacin hydrochloride (CIP), beclomethasone dipropionate (BP), gatifloxacin hydrochloride (GAT) and ambroxol hydrochloride (AMB), trans-resveratrol, budesonide (BD) were supplied from Junda Pharmaceutical Co. Ltd. (Changzhou, China). Meropenem trihydrate (MEM) and tigecycline (TIG) were purchased from Afine Chemical (Hangzhou, Zhejiang, China). Rifampicin (RIF) was obtained from Leshan Sanjiu-Longmarch Pharmaceuticals Co., Ltd. Disodium hydrogen phosphate, phosphoric acid, L-Leucine (LEU), mucin, egg yolk emulsion, deoxyribonucleic acid (DNA) from fish sperm, casein hydrolysate, diethylenetriamine pentaacetic acid (DTPA), NaCl, KCl, mannitol, colistin sulfate (COL), ammonium acetate, trifluoroacetic (TFA), curcumin (CUR) and quercetin (QUER) were purchased from Sigma Chemical Co. (Louis, MO, USA). Ultrapure water was used in the experiments. HPLC grade acetonitrile, molecular biology grade of 1M Tris buffer (pH 8.0), methanol (MeOH) and acetonitrile (ACN) was supplied by Merck (Darmstadt, Germany). Dimethyl sulfoxide (DMSO) was from Sigma (Poole, UK). Prospan[®] was from Engelhard Arzneimittel GmbH & Co. KG. GM-CSF Elisa Kit and IL-1 β were from Abcam (Cambridge, MA, USA). Etest strips were purchased from bioMérieux SA (Marcy-l'Etoile, France). RPMI, fetal bovine serum (FBS) and DPBS were from Gibco (Paisley, Scotland). T25 culture flask was from Thermo Scientific Inc (Rochester, NY, USA) and Eppendorf tubes were from Eppendorf North America, Inc (Westbury, NY, USA). Mueller-Hinton broth

(Oxoid, Basingstoke, UK) was used as the culture media for the antimicrobial activity test. *Klebsiella pneumoniae* ATCC 12885, *Pseudomonas aeruginosa* ATCC 90207 and *Staphylococcus aureus* ATCC 4330 were obtained from the National University Hospital (Singapore) and used as the test microorganism. Clinical isolates of multidrug resistant bacteria used in the study were obtained from the National University Hospital, Singapore and included *Pseudomonas aeruginosa* PSE2 (resistant to ciprofloxacin, aminoglycosides and carbapenems), *Klebsiella pneumoniae* ENT443 (resistant to ciprofloxacin and carbapenems), *Escherichia coli* EC424 (resistant to ciprofloxacin and carbapenems) and *Acinetobacter baumannii* AB1 (resistant to ciprofloxacin, aminoglycosides and carbapenems). A549 cell line was from the American Type Culture Collection (ATCC) (Manassas, VA, USA).

2.2. Preparation of spray-dried powders

2.2.1. Preparation of SD-CIP, SD-BP and SD-CIP/BP

Powders of ciprofloxacin hydrochloride (SD-CIP), beclomethasone dipropionate (SD-BP), and binary combination powders of beclomethasone dipropionate/ciprofloxacin hydrochloride in a weight ratio of 1:32.5 (SD-CIP/BP) were prepared by spray drying CIP alone, BP alone and CIP with BP from a methanol-water co-solvent (3.5:1 v/v) feedstock at a feed concentration of 0.7% (w/v) using a B-90 Nano Spray Dryer (Büchi Labortechnik AG, Flawil, Switzerland) [1-3] with the following parameters: spray mesh size 7 µm; inlet temperature 75°C (outlet temperature 38-42°C); nitrogen flow rate 120 L/min and a relative spray rate 4 mL/h. All solutions were filtered through a 0.45 µm

syringe filter (Millipore, Bedford, MA, USA) prior to spray-drying to minimize blockage due to any undissolved particles at the spray mesh. The spray-dried powders were stored in a desiccator at room temperature for further characterization.

2.2.2. Preparation of SD-CIP, SD-GAT, SD-AMB, SD-CIP/GAT/AMB and SD-CIP/GAT/AMB/LEU

Powders of ciprofloxacin hydrochloride (SD-CIP), gatifloxacin hydrochloride (SD-GAT), ambroxol hydrochloride (SD-AMB), ternary combination powders of ciprofloxacin hydrochloride/gatifloxacin hydrochloride/ambroxol hydrochloride with (SD-CIP/GAT/AMB/LEU) and without leucine (SD-CIP/GAT/AMB), SD-CIP/GAT and SD-AMB/LEU were obtained by spray drying their aqueous solutions on a B-290 Mini Spray Dryer (Büchi Labortechnik AG, Flawil, Switzerland) with the following operating parameters: inlet temperature of 190°C (outlet temperature 100°C), atomization rate of 670 L/h, aspiration rate of 57.6 m³/h and feed rate of 3.5 mL/min. The feed solution concentration was maintained at 0.8% (w/v) for all the formulations. The ratio of CIP:GAT:AMB in the ternary formulations (both with and without leucine) was 1:2.5:5.5 [4]. For SD-CIP/GAT/AMB/LEU and SD-AMB/LEU, the leucine content was 13% (w/w). The ratio of CIP:GAT for SD-CIP/GAT was maintained at 1:2.5. The spray-dried powders were stored in a desiccator at room temperature for further characterization.

2.2.3. Preparation of SD-COL, SD-MEM, SD-RIF, SD-TIG, SD-COL/MEM/RIF, SD-COL/MEM/TIG and SD-COL/TIG/RIF

Powders of colistin (SD-COL), meropenem (SD-MEM), rifampicin (SD-RIF), tigecycline (SD-TIG), ternary combination powders of colistin/meropenem/rifampicin (SD-COL/MEM/RIF) in weight ratio of 5:4:12, colistin/meropenem/tigecycline (SD-COL/MEM/TIG) in weight ratio of 5:4:4 and colistin/tigecycline/rifampicin (SD-COL/TIG/RIF) in weight ratio of 5:4:12 were prepared by spray drying the ethanol-water co-solvent (2:1 v/v) feedstock of antimicrobial agent/s on a B-90 Nano Spray Dryer [2-5] (Büchi Labortechnik AG, Flawil, Switzerland) with the following operating parameters: spray mesh size 5.5 μm ; inlet temperature 120°C (outlet temperature 60-65°C); nitrogen flow rate 120 L/min and a relative spray rate 4 mL/h. The feed solution concentration was maintained at 0.65% (w/v) for all the formulations. In this work, in vitro susceptibility of control and MDR strains against each individual drug of the different combinations was first determined. The MIC values obtained experimentally were further guided by EUCAST (European Committee on Antimicrobial Susceptibility Testing) clinical susceptibility breakpoints [6] to obtain ratios that would be ‘universally’ potent. All solutions were filtered through a 0.45 μm syringe filter (Millipore, Bedford, MA, USA) prior to spray-drying to minimize blockage due to any undissolved particles at the spray mesh. The spray-dried powders were stored in a desiccator at room temperature for further characterization.

2.2.4. Preparation of SD-CUR/QUER/RES, SD-CUR/QUER/RES/LEU-25 and SD-CUR/QUER/RES/LEU-50

Ternary combination powders of curcumin/quercetin/trans-resveratrol in weight ratio of 1:1:1 with (SD-CUR/QUER/RES-LEU) and without leucine (SD-CUR/QUER/RES) were prepared by spray drying the feed solution (ethanol:water = 3:1 v/v co-solvent system) on a B-90 Nano Spray Dryer (Büchi Labortechnik AG, Flawil, Switzerland). The L-leucine contents in the spray-dried powders were 0% (SD-CUR/QUER/RES), 25% (SD-CUR/QUER/RES/LEU-25) and 50% (SD-CUR/QUER/RES/LEU-50) (w/w) of the total solid content. The feed concentration was maintained at 0.6% (w/v) regardless of the content of L-leucine and spray dried at the following conditions: spray mesh size 7 μm ; inlet temperature 90°C (outlet temperature 40-45°C); nitrogen flow rate 120 L/min and a relative spray rate 4 mL/h. All solutions were filtered through a 0.45 μm syringe filter (Millipore, Bedford, MA, USA) prior to spray-drying to minimize blockage due to any undissolved particles at the spray mesh. The spray-dried powders were stored in a desiccator at room temperature for further characterization.

2.3. Surface morphology

The morphology of the powder particles was examined by field emission scanning electron microscopy (FESEM, JEOL JSM-6700) at 5 kV. Prior to imaging, the samples were dispersed onto carbon sticky tabs and coated with gold for 100 s using a sputter coater (Cressington 208HR, Watford, UK).

2.4. Particle size analysis

The particle size distribution of the spray-dried powders was determined by laser diffraction using Malvern Mastersizer 2000 (Malvern Instruments, UK) using the Scirocco dry dispersion unit. The spray-dried powders were dispersed in triplicates at 3 bars of pressure and with refractive index (RI) ranging from 1.4 to 1.8.

2.5. Powder crystallinity

Powder crystallinity of the samples was assessed by powder X-ray diffraction (pXRD) at room temperature using an X-ray diffractometer (D8 Advance; Bruker AXS GmbH, Karlsruhe, Germany). Samples were scanned from 2-50° (2 θ) at an angular increment of 0.04° and at 1 s per step using Cu K α radiation generated at 35 kV and 40 mA.

2.6. Surface composition evaluation

The surface elemental composition of the powdered samples was measured using X-ray photoelectron spectroscopy (XPS) on a VG ESCALAB 250 spectrometer (Thermo Electron, U.K.) that was equipped with a non-monochromatized Al α X-ray source (1486 eV). Measurements were recorded for a 20 eV pass energy, a 0.1 eV kinetic energy step and a 0.05 s dwelling time. The powders were pressed onto double sided conductive adhesive tapes. Experimental molar percentages of all elements except hydrogen were derived

from the XPS peak areas as described elsewhere [7]. Elemental weight percentages (Wt. %) were obtained via multiplying the molar percentages (At. %) with the respective atomic mass.

2.7. Powder dissolution

Two hundred milliliter dissolution medium (0.2% w/v sodium dodecyl sulphate solution) at $37.0 \pm 0.5^{\circ}\text{C}$ was passed through a 25 mm i.d. flow-through cell (Millipore Swinnex filter holders, USA, utilizing a Pall HT Tuffryn 0.2 μm membrane disc filters, USA) containing 20 mg of sample, and re-circulated in a closed-loop configuration at 1.6 mL/min using a peristaltic pump (pump speed stability of $\pm 0.5\%$) (Gilson MiniPuls3, USA) [8, 9]. At regular time intervals, 0.7 mL samples were withdrawn, diluted with 0.5 mL ethanol and then analyzed for their concentrations with the corresponding HPLC methods. Studies were carried out in triplicate.

2.8. Surface area determination

The specific surface area of the spray-dried powders was determined by a surface area analyzer (Micromeritics ASAP 2420, USA), using nitrogen as the adsorbate gas. Samples were degassed at 25°C for 24 h on a vacuum dryer, prior to the measurements. The specific surface area was then determined by the multipoint Brunauer–Emmett–Teller (BET) gas adsorption/desorption method.

2.9. Thermogravimetric analysis (TGA)

TGA studies were carried out using a thermogravimetric analyzer (TGA Q500, TA Instruments, USA). Samples were weighed into a platinum pan and heated in flowing air (90 mL/min) from room temperature to 800°C at a scan rate of 10°C/min. Moisture content (%) was determined from the stable weight loss (%) that occurred at about 100°C [10].

2.10. Dynamic vapour sorption (DVS)

Moisture sorption isotherms were obtained using the gravimetric vapour sorption (DVS Advantage 1, Surface Measurement Systems, UK) technique. Samples were weighed into a round-bottomed quartz sample pan and exposed to sorption desorption cycles from 0 to 90% RH at 25°C and steps of 10% RH. Equilibrium moisture content at each humidity step was determined by a dm/dt of 0.02% per minute [11].

2.11. Focused-ion-beam (FIB) milling

The internal morphology and structure of the particles was analysed using a focused-ion-beam/scanning electron microscope dual beam system (Nova Nanolab 200 FIB/SEM system, FEI Corp., Hillsboro, OR). Briefly, the powder samples were sprinkled onto a 100-mesh transmission electron microscopy (TEM) grid (GCu100, ProSciTech, Australia) and then mounted onto the stage with a thin layer of double-sided adhesive carbon tape before being sputter coated with gold at 20 mA for 120 s (JEOL JFC-1600 Auto Fine Coater, Tokyo,

Japan). While undergoing FIB milling (using a 30 kV accelerating voltage with the electron beam current of 100 pA), the cross-section of particles were concurrently visualized under SEM at 5 kV [12].

2.12. Powder dispersion

2.12.1. In vitro aerosol performance of spray-dried powders in a multi-stage liquid impinger (MSLI)

The aerosol performance of SD-CIP, SD-BIP and SD-CIP/BP (spray-dried powders from Chapter 3), SD-CIP, SD-GAT, SD-AMB, SD-CIP/GAT/AMB and SD-CIP/GAT/AMB/LEU (spray-dried powders from Chapter 4), and SD-CUR/QUER/RES, SD-CUR/QUER/RES/LEU-25, SD-CUR/QUER/RES/LEU-50 (spray-dried powders from Chapter 6) was assessed using a Multi-Stage Liquid Impinger (MSLI, Copley Scientific, Nottingham, UK) coupled with a United State Pharmacopoeia (USP) stainless steel throat. The method followed the procedure specified for DPIs in the British Pharmacopoeia [13]. Prior to testing, 20 mL of co-solvent or deionized water (methanol:water = 3.5:1 v/v for spray-dried powders from Chapter 3; deionized water for spray-dried powders from Chapter 4 and ethanol:water = 3:1 v/v for spray-dried powders from Chapter 6) was added across all four stages of the MSLI. Approximately 20 ± 2 mg of powder from each formulation was filled into a hydroxypropyl methylcellulose (HPMC) capsule (size 3, Capsugel[®], NJ, USA), loaded into the high efficiency Aerolizer[®] inhaler (Novartis Pharmaceuticals, Basel, Switzerland) [14] and pierced. The powder was then dispersed for 4 s at 60 L/min and 2.4 s at 100 L/min. The flow through the MSLI was measured using

a calibrated flow meter (TSI Model 4040C, TSI Instrument Ltd., Buckinghamshire, UK), controlled by a high capacity vacuum pump (Model HCP5, Copley Scientific, Nottingham, UK) and a critical flow controller (TPK 2000, Copley Scientific, Nottingham, UK). After dispersion, the device, capsule, throat and each stage of the MSLI were washed separately and thoroughly using corresponding co-solvents or deionized water. The solutions were then assayed by high performance liquid chromatography (HPLC) after appropriate sample dilutions. The aerodynamic cut-off diameters of stages 1, 2, 3 and 4 are 13.0, 6.8, 3.1 and 1.7 μm at a flow rate of 60 L/min and 10.07, 5.27, 2.4 and 1.32 μm at a flow rate of 100 L/min. Particles with diameter size less than 1.7 (flow rate = 60 L/min) and 1.32 μm (flow rate = 100 L/min) were captured on an integral glass fibre filter paper (Pall Corporation, New York, USA).

2.12.2. In vitro aerosol performance of spray-dried powders in a Next Generation Impactor (NGI)

The aerosol performance of SD-COL, SD-MEM, SD-RIF, SD-TIG, SD-SD-COL/TIG/RIF, SD-COL/MEM/RIF and SD-COL/MEM/TIG (spray-dried powders from Chapter 5) was assessed using a Next Generation Impactor (NGI, Copley Scientific, Nottingham, UK) coupled with a United States Pharmacopoeia (USP) stainless steel throat. The method followed the procedure specified for DPIs in the British Pharmacopoeia [4, 15]. Prior to testing, all the eight impactor stages were sprayed with MOLYKOTE[®] 316 silicone grease release spray (Dow Corning Corp., Midland, MI) to minimize particle bounce. Approximately 10 mg of powder from each formulation was filled into a hydroxypropyl methylcellulose (HPMC) capsule (size 3, Capsugel[®], New

Jersey, USA), loaded into an Aerolizer[®] inhaler (Novartis Pharmaceuticals, Basel, Switzerland), pierced and then actuated for 4 s at 60 L/min. The flow through the NGI was measured using a calibrated flow meter (TSI Model 4040C, TSI Instrument Ltd., Buckinghamshire, UK), controlled by a high capacity vacuum pump (Model HCP5, Copley Scientific, Nottingham, UK) and a critical flow controller (TPK 2000, Copley Scientific, Nottingham, UK). After actuation, the device, capsule, throat and each part of the NGI were washed separately and thoroughly using ethanol-water co-solvent. The solutions were then assayed by high performance liquid chromatography (HPLC) after appropriate sample dilutions. At 60 L/min, the aerodynamic cut-off diameters of stages 1, 2, 3, 4, 5, 6 and 7 are 8.06, 4.46, 2.82, 1.66, 0.94, 0.55 and 0.34 μm . Particles with diameter size less than 0.34 μm were captured on a micro orifice collector (MOC) beyond stage 7.

Each dispersion test (both MSLI and NGI) was performed in triplicates to obtain mean values. Dispersion was carried out in a walk-in environmental chamber (Synergys, Singapore) maintained at 25°C and 40% RH. In this study, fine particle fraction (FPF) represents the mass fraction of drug particles smaller than 5 μm in the aerosol cloud relative to the total mass recovered and was obtained by interpolation to the cumulative percent undersize at 5 μm . FPF(emitted) was obtained when the fine particle dose was expressed relative to the emitted dose. The mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) were calculated from the log-probability plot of the MSLI results. MMAD is the equivalent aerodynamic diameter at 50% cumulative mass of particles and GSD is the ratio of aerodynamic diameters at 84% and 50% cumulative mass.

2.13. Drug content quantification

Drug contents in the spray-dried powders and in the aliquots collected from the MSLI and NGI were analyzed via HPLC (1100 series, Agilent Technologies, CA, USA). The lack of additional peaks in all chromatograms suggested the absence of chemical degradation due to the spray-drying process.

2.13.1. Quantification of SD-CIP, SD-BP and SD-CIP/BP

For ciprofloxacin hydrochloride assays, a Zorbax SB C-18 column (4.6 mm x 150 mm, 3.5 μ m) (Agilent Technologies, CA, USA) was used as the stationary phase (column temperature 25°C), and a mixture of 0.025M disodium hydrogen phosphate buffer (adjusted to pH 3.0 with phosphoric acid) and acetonitrile (80:20, v/v) as the mobile phase. The flow rate was operated at a constant 1 mL/min and the UV wavelength for detection was 293 nm. For the assay of beclomethasone dipropionate, the mobile phase consisted of acetonitrile/water (60:40, v/v) delivered at 1.5 mL/min under a UV detection wavelength of 250 nm. The retention times of ciprofloxacin hydrochloride and beclomethasone dipropionate were 2.3 min and 4.7 min, respectively.

2.13.2. Quantification of SD-CIP, SD-GAT, SD-AMB, SD-CIP/GAT/AMB and SD-CIP/GAT/AMB/LEU

The quantification of ciprofloxacin hydrochloride, gatifloxacin hydrochloride and ambroxol hydrochloride were conducted using the same HPLC assay method for ciprofloxacin hydrochloride in Section 2.13.1, but an UV absorbance wavelength of 245 nm were employed for the simultaneous detection of ciprofloxacin hydrochloride, gatifloxacin hydrochloride and

ambroxol hydrochloride. The retention times of ciprofloxacin hydrochloride, gatifloxacin hydrochloride and ambroxol hydrochloride were 3.1, 5.1 min and 10.1 min, respectively.

2.13.3. Quantification of SD-COL, SD-MEM, SD-RIF, SD-TIG, SD-COL/MEM/RIF, SD-COL/MEM/TIG and SD-COL/TIG/RIF

Assay of colistin was conducted via a modified HPLC method originally described by Bai et. al. [16] with Zorbax SB C-18 column (4.6 mm x 150 mm, 3.5 μ m) (Agilent Technologies, CA, USA) as the stationary phase. The mobile phase consisted of 0.05% (v/v) TFA aqueous solution (solvent A) and acetonitrile (solvent B), delivered at 1 mL/min under a linear gradient: 0-8 min, 80% to 64% A; 8-10 min, 64% A to 80% A. Colistin peak appeared at a retention time of 5.3 min under a UV wavelength of 214 nm. A Zorbax Extend C-18 column (4.6 mm x 150 mm, 3.5 μ m) (Agilent Technologies, CA, USA) was used as the stationary phase for simultaneous assays of meropenem, tigecycline and rifampicin using a modified method originally described by Bradford et. al. [17]. The chromatograph separation was performed using a gradient mobile phase system starting with 95:5 solvent A (95% 50 mM ammonium acetate, pH 6.2–5% acetonitrile): B (5% 50 mM ammonium acetate, pH 6.2–95% acetonitrile) changed to 50:50 solvent A:B over 10 min then ramped to 5:95 solvent A:B in 1 min. A 2-min isocratic hold was followed by a step back to the initial conditions. A UV absorbance wavelength of 244 nm was employed for the simultaneous detection of meropenem, tigecycline and rifampicin at retention times of 1.7, 5.7 and 10.6 min, respectively.

2.13.4. Quantification of SD-CUR/QUER/RES, SD-CUR/QUER/RES/LEU-25 and SD-CUR/QUER/RES/LEU-50

The quantification of trans-resveratrol, quercetin and curcumin was performed using a Zorbax SB C-18 column (4.6 mm x 150 mm, 3.5 µm) (Agilent Technologies, CA, USA) as the stationary phase. Assay of trans-resveratrol was conducted via a modified HPLC method originally described by Souto et. al. [18] an isocratic elution (1.2 mL/min flow rate) using a H₂O:ACN (75:25, v/v) solution (acidified to pH 3.0 using phosphoric acid) as mobile phase. Trans-resveratrol peak appeared at a retention time of 5.6 min under a UV wavelength of 306 nm. Analysis of quercetin was performed by the modified method reported by Agilent [19]. Quercetin was separated using a mobile phase consisted of a mixture of 0.5% phosphoric acid and MeOH (40:60, v/v) at a flow rate of 1 mL/min. The detection wavelength and retention time of quercetin were 370 nm and 3.0 min, respectively. Elution of curcumin was carried out using a mobile phase made up of a mixture of 50 mM phosphate buffer that acidified to pH 3.5 and ACN (40:60, v/v) at a flow rate of 0.7 mL/min. The peak of curcumin was eluted at 5.0 min and detected by its UV absorbance at 430 nm.

2.14. Antimicrobial activity of spray-dried powders

2.14.1. Determination of antimicrobial minimum inhibitory concentration (MIC) of SD-CIP, SD-BP and SD-CIP/BP

The in vitro activity of SD-BP, SD-CIP and SD-BP/CIP was tested against bacterial type strains of *Klebsiella pneumoniae* ATCC 12885, *Pseudomonas*

aeruginosa ATCC 90207 and *Staphylococcus aureus* ATCC 4330. The microdilution test was performed in accordance to the Clinical and Laboratory Standards Institute (CLSI) procedures [20] using a final bacterial inoculum of approximately 5×10^5 (colony forming units) CFU/ml in a sterile 96-well plate bearing two-fold drug dilutions. The MIC was defined as the lowest drug concentration that inhibited the visible growth of microorganisms after an overnight incubation [21]. The MIC assays were carried out in duplicate for each strain.

2.14.2. Antimicrobial activity of SD-CIP/GAT/AMB/LEU spray-dried powder

2.14.2.1. Preparation of artificial sputum medium (ASM)

The artificial sputum medium comprising largely of mucin, DNA, amino acids and salts was prepared as according to Haley et. al. [22] with modifications to sterilizing the medium. Briefly, a solution of fish sperm DNA, salts (NaCl and KCl), casein hydrolysate and DTPA adjusted to pH 6.8 was filter sterilized through a 0.22 μ m membrane filter. Mucin solution prepared separately was adjusted to pH 6.8, autoclaved, and then combined with the fish sperm DNA, casein hydrolysate and salt solution. Finally, sterile egg yolk emulsion was added to the entire contents thus making up the complete artificial sputum medium.

2.14.2.2. Preparation of *Pseudomonas aeruginosa* spiked media

Pseudomonas aeruginosa spiked ASM was prepared as follows: Overnight cultures of *Pseudomonas aeruginosa* ATCC 90207 were diluted in Mueller

Hinton Broth (MHB) to an optical density measured at wavelength 600 nm (OD₆₀₀) of 0.01. This suspension was then further diluted 1:100 in fresh ASM media for use in antibacterial assays.

2.14.2.3. Dose-response killing of SD-CIP/GAT/AMB/LEU

The efficacy of the SD-CIP/GAT/AMB/LEU drug combination against *Pseudomonas aeruginosa* ATCC 90207 was tested in the ASM media (benchmarked against SD-CIP/GAT and SD-AMB/LEU controls). Drug concentrations for testing started at 2/5.2/11.4 µg/ml (CIP/GAT/AMB) and two-fold dilutions of this were made in ASM media until a final concentration of 0.0039/0.010/0.022 µg/ml was reached. The experiments were set up in 24 well plates using a total of 1 ml of *Pseudomonas aeruginosa* spiked media per well. The drug combination was incubated with spiked media at 37°C for 24 h after which CFU counts were performed. The experiment was carried out in triplicates.

2.14.3. Antimicrobial activity of ternary formulation of colistin combinations

2.14.3.1. Determination of antimicrobial minimum inhibitory concentration (MIC) of SD-COL, SD-MEM, SD-TIG and SD-RIF

The Etest is a plastic strip with a predefined antibiotic gradient immobilized on one side and a MIC interpretive scale printed on the other side. The procedure for MIC determination was carried out as described by the Etest strip manufacturer (bioMérieux, Marcy-l'Etoile, France). Briefly, bacterial

suspensions with a turbidity equivalent to McFarland standard 0.5 were swabbed evenly onto Mueller Hinton agar plates with a sterile cotton swab. After the surfaces of the plates were dried, the Etest strips of all the tested antimicrobial agents were applied. The plates were incubated at 37°C for 20 h. The MIC value was determined as the value at which there was complete inhibition of growth (i.e. the point where an elliptical zone of growth inhibition intersects the scale). The MIC values were interpreted as susceptible, intermediate or resistant as according to the EUCAST guidelines for susceptibility testing.

2.14.3.2. Time-kill studies of ternary formulation of colistin against different MDR pathogenic respiratory bacteria

In vitro antimicrobial activity of the spray-dried powders was determined quantitatively via time-kill studies. Overnight cultures of the test organism was diluted in Mueller-Hinton broth (MHB) to give a starting bacterial density of approximately 5×10^5 CFU/ml [4]. Spray-dried powders were added to the cultures such that the final concentrations were at 1 x MIC (concentration was chosen to be clinically relevant and achievable in vivo; colistin is known to exhibit very active killing of gram-negative bacteria (even at low doses of 0.5 x MIC) [23, 24], hence the choice to test the drug combinations at 1 x MIC would seem appropriate). The cultures were then incubated at 37°C with shaking (200 rpm). Bacterial cell counts were estimated at time 0 and 24 h. Synergy was defined as a ≥ 2 log₁₀ decrease in colony count after 24 h by the combination compared to the most active single agent, additivity as a 1 to < 2 log₁₀ decrease in colony count after 24 h by the combination compared to the most active

single agent and indifference as a $<1 \log_{10}$ increase or decrease in colony count after 24 h by the combination compared with that by the most active single agent [25]. All microbiological work was carried out under biosafety level 2 (BSL-2) conditions.

2.15. Measurement of GM-CSF in cell culture medium

Carcinoma human alveolar basal epithelial cell line A549 were grown till 80% confluency in RPMI (Gibco #11875) media supplemented with 5% Fetal Bovine Serum. (Gibco #10082). The cells were then seeded into T25 filter capped tissue culture flasks (Thermo #156367) with a seeding density of 4×10^5 cells/ml in a total volume of 2 mL. A flask was prepared for each individual drug sample. The cells were left to incubate at 37°C and 5% CO₂ for 24 h. The spent media was removed and the cell monolayer was then washed with DPBS (Gibco #14190) of fresh serum-free RPMI was added to the flask and cultured for a further 24 h. After this step, the cells should have reached 90% confluency. Lyophilized IL-1B recombinant protein (Abcam #9617) was reconstituted to a stock solution. The stock solution of IL-1B was dosed with the cells to produce a final IL-1B concentration of 1ng/ml. The drug samples were also concurrently dosed into the cells. The cells were left to incubate at 37°C and 5% CO₂ for 24 h. After the dosage period, 1ml of the cell culture supernatant was removed from the flask and pipetted into Eppendorf tubes. The supernatant was then centrifuged at 1000 rpm x 5 min to remove any cell debris. The decanted liquid supernatant was kept on ice to be measured for its GM-CSF levels. The ELISA GM-CSF kit with its reagents was prepared according to the manufacturer's

instructions (Abcam #100529). A standard curve of GM-CSF was determined by preparing a range of GM-CSF concentrations from 2.05 pg/ml to 500 pg/ml. The samples and standards were prepared in duplicates for the ELISA assay according to the supplied kit. The levels of GM-CSF in the samples were determined from reading off the absorbance values against the standard curve.

2.16. References

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Chapter 3

Steroid-Decorated Antibiotic Microparticles for Inhaled Anti-Infective Therapy

3.1. Introduction

Community-acquired pneumonia (CAP) is an inflammatory condition of the lungs due to an infection arising from sources external to a hospital or extended-care facility (e.g. nursing home) setting. Although CAP can be brought about by bacteria, viruses or fungi, the most common cause of CAP in adults is still via a bacterial infection. The severity of CAP has been linked to the extent of inflammation in the body, as inflammation is the body's natural response to the invading infection-causing pathogens [1, 2]. Despite advances in vaccination and antimicrobial therapy, CAP remains as a leading cause of human morbidity and mortality, and has afflicted the world population with a huge socio-economic cost [1-4].

Chronic obstructive pulmonary delivery (COPD) is a pulmonary condition where the airways become chronically inflamed and irreversibly narrowed, hence leading to a shortness of breath. Furthermore, patients with COPD appear to be at a higher risk of developing CAP than patients in the general population [5, 6]. Recent studies have reported on the impaired immunological mechanisms in COPD patients brought about by the defective alveolar macrophage phagocytosis of bacteria. This consequently increases the susceptibility of COPD patients to infections [7, 8]. Moreover, COPD patients tend to develop persistent bacterial colonization in the lower airways through

the inflammatory mechanism, hence leading to exacerbations of COPD and progression of airway obstruction [9, 10]. Therefore, CAP patients with COPD frequently experience poorer clinical outcomes than patients without COPD. The former has been reported to exhibit higher 30- and 90-day mortality than the latter [11]. As these risks tend to increase with COPD severity, it is vital for CAP patients with COPD to seek medical intervention early to prevent COPD exacerbations and limit disease progression.

Currently, antibiotic treatment remains the mainstay of CAP therapy. However, patients with obstructive airways disease complicated by CAP could also benefit from the inclusion of inhaled corticosteroids into their treatment regimen in view of the drug's potent immunomodulatory and anti-inflammatory properties [3]. Although the release of inflammatory mediators from alveolar macrophages is useful for eliminating invading pathogens from pulmonary infections, excessive releases are on the contrary, harmful to the lungs. Hence, modulation of the inflammatory response is vital to creating a balance between the beneficial and harmful effects [12-14].

Corticosteroids are the most commonly used physiological inhibitors for inflammation. They interfere with the inflammatory expression and action that are associated with COPD and pneumonia. The inflammatory response in the lung is a complex process that involves the coordinated expression of both pro-inflammatory and anti-inflammatory cytokines [1]. Corticosteroids can switch off genes that encode pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF- α), interleukin 1 beta (IL-1 β) and interleukin-6 (IL-6), and switch on those that encode anti-inflammatory cytokines such as IL-10 [15-17]. For

CAP patients with COPD, the down-regulation of the unwanted excessive cytokine response by corticosteroids has the beneficial effect of reducing excessive pulmonary inflammation and accelerating clinical recovery. In addition, bronchospasms (wheezing), a common condition found in these patients, could also be treated with the corticosteroids [3, 18, 19].

Understandably, clinicians have therefore applied the use of corticosteroids as an adjunctive therapy (i.e. anti-inflammatory properties) for different infectious diseases as early as the 1950s [20]. The use of corticosteroids in combination with antibiotics has been proven to be effective and safe in several randomized controlled trials in patients with bacterial and tuberculous meningitis [21-23], tuberculous pericarditis [24], septic arthritis [25] and septic shock [26]. In CAP-COPD patients, co-administration of corticosteroids with antibiotics has been shown to be safe and efficacious, and is therefore frequently administered by the physician for treatment [2, 3]. On a further note, for smokers who are predisposed to COPD and bacterial infections (i.e. tobacco smoke compromise the anti-bacterial function of leukocytes, including neutrophils, monocytes, T cells and B cells) [6], treatment via the steroid-antibiotic combination will no doubt be a useful and timely therapeutic intervention as well.

Most of the material in this chapter was published in *Journal of Pharmaceutical Sciences* [27]. Some modifications to the text are included here.

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3.2. Aim

The objective of this study is to pioneer, via state-of-the-art nanospray drying [28, 29], a novel inhalable dry powder formulation incorporating both an antibiotic and a corticosteroid for the treatment of bacterial infections in CAP-COPD patients.

Ciprofloxacin HCl (CIP) and beclomethasone dipropionate (BP) were selected as the model antibiotic and corticosteroid in view of their broad-spectrum activity against both Gram-negative and Gram-positive bacteria (especially in CAP [4]), and robust anti-inflammatory activity [30], respectively. Among the members of the fluoroquinolone family (a class of broad-spectrum antibiotics), the antibiotic ciprofloxacin has been known for its clinical efficacy and low potential for adverse effects [31-33]. In addition, the efficacy, safety and tolerability of ciprofloxacin DPI in human subjects had been successfully evaluated under Phase I and II clinical trials [33, 34]. Phase III (RESPIRE programme) efficacy studies are currently underway [35]. Inhaled BP, being a robust anti-inflammatory agent, was previously demonstrated to be an effective treatment for patients with non-asthmatic chronic airflow obstruction [36].

The feasibility of combining the antibiotic and the corticosteroid as an inhalable dry powder formulation for direct concomitant delivery to the lung was investigated. The spray-dried binary formulation that contained CIP and BP (SD-CIP/BP), as well as their single counterparts (SD-CIP and SD-BP) were evaluated for their physicochemical characteristics, aerosol performance and antimicrobial properties.

3.3. Results and discussion

3.3.1. Preparation of spray-dried powders

Spray-dried powders of corticosteroid alone (SD-BP), antibiotic alone (SD-CIP) and binary combination of corticosteroid and antibiotic (SD-BP/CIP) were successfully prepared and collected at yields of more than 80% using the Nano Spray Dryer B-90 [29]. High product yield is readily achieved on the Nano Spray Dryer B-90, with published reports listing it as between 70% and 90% [28, 37-39].

CIP is a water-soluble antibiotic whereas BP is a poorly-water soluble steroid that can only be dissolved in organic solvents such as methanol, chloroform or acetone. In order to co-spray dry both hydrophilic and hydrophobic active pharmaceutical ingredients (APIs) simultaneously for the production of particles with uniform drug composition, co-solvent selection and ratio are both very important aspects. In this work, a water-methanol mix was selected as the co-solvent system for BP and CIP in view of their mutual miscibility and low toxicity. The water-methanol co-solvent volume ratio was adjusted to 1:3.5 to ensure good solubility of both APIs in the co-solvent system with no precipitation.

Unlike oral corticosteroids which must travel throughout the patient's body to reach the lung, inhaled corticosteroids are typically used in much smaller doses (i.e. a few hundred micrograms) as there is direct lung-targeting [40]. Furthermore, the maximum daily dose of inhaled BP has been recommended to be at approximately 1 mg to avoid significant reduction in plasma cortisol concentrations [41, 42].

Antibiotics are widely used to treat bacterial infections by either killing (bactericidal) or inhibiting the growth (bacteriostatic) of bacteria. It is usually used at high concentrations to ensure good tissue penetration and maximum bacterial kill [43, 44]. Currently, Cipro Inhale[®], an inhaled antibiotic dry powder formulation undergoing Phase III clinical trial, has been found to be safe and well-tolerated (via Phase I and II trials), when applied at a single inhaled dose of 32.5 mg [33, 34].

In this work, the binary dry powder formulation containing BP and CIP was prepared at 1: 32.5 weight ratio (adapted from reported clinical dosing). Although the weight ratio of BP was as low as 3% in the binary formulation, it was still well-preserved (as close as 100% in comparison to the feed, Table 3.1.) in the final spray-dried powder.

Table 3.1. Composition of the spray-dried particles (drug contents measured by HPLC)^a

Formulation n	Drug Content (w/w %)				% of Ideal (Relative to feed)	
	Feed		Actual			
	BP	CIP	BP	CIP	BP	CIP
SD-BP	100.0	-	99.7 ± 0.5	-	99.7 ± 0.5	
SD-CIP	-	100.0	-	99.3 ± 0.7		99.3 ± 0.7
SD-BP/CIP	3.0	97.0	2.9 ± 0.1	97.7 ± 0.8	96.7 ± 3.3	100.7 ± 0.8

Mean ± standard deviation (n=3)

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3.3.2. Surface morphology and particle size

The surface morphologies of the single and binary spray-dried particles are shown in Figure 3.1. All the spray-dried particles were generally spherical in geometry, with the SD-CIP having a smoother surface (Figure 3.1c & 3.1d)

than the SD-BP (i.e. slightly corrugated surface) (Figure 3.1a & 3.1b). The surface morphology of SD-BP/CIP (Figure 3.1e & 3.1f) was found to be closer to that of SD-BP, whereby a slightly corrugated surface was obtained. This observation was not surprising, even though CIP was the major component of the powder (i.e. 97% w/w CIP vs 3% w/w BP). The final distribution of each solute component in the spray-dried particle would very much depend on the solutes' surface activities (which leads to the preferential adsorption of components on the sprayed droplet surface) and diffusivities in the spray solution. In the present binary co-solvent system (i.e. methanol-water), the saturation of hydrophobic API (BP) occurred much faster than that of the hydrophilic API (CIP) due to the preferential evaporation of methanol over water and the associated changes in methanol-water ratio during the drying process. This then led to the accumulation and precipitation of the hydrophobic component at the liquid-air interface. Consequently, more hydrophobic component is found on the surface of the dried particles [45-47]. Thus, this explains the similar surface morphologies of both SD-CIP/BP and SD-BP. The XPS results discussed under Section 3.3.4 further supports this finding.

Figure 3.1. FESEM images of spray-dried powders. (a) SD-BP, (b) SD-BP (close-up view), (c) SD-CIP, (d) SD-CIP (close-up view), (e) SD-BP/CIP and (f) SD-BP/CIP (close-up view). Reproduced with permission from Ref. [27]. Copyright 2014 Elsevier

As all the single and binary spray-dried powders were prepared under the same spray-drying conditions, they were thus found to have comparable particle size and span (Table 3.2.). The particle size analysis revealed that all the spray-dried powders were within the respirable size range, with a d_{50} of $2.21 \pm 0.07 \mu\text{m}$,

$2.36 \pm 0.02 \text{ }\mu\text{m}$ and $2.36 \pm 0.01 \text{ }\mu\text{m}$ for SD-BP, SD-CIP and SD-BP/CIP, respectively.

Table 3.2. Volume particle size distribution of the spray-dried powders^a

	SD-BP	SD-CIP	SD-BP/CIP
$d_{10} \text{ (}\mu\text{m)}$	1.01 ± 0.01	0.93 ± 0.01	0.88 ± 0.06
$d_{50} \text{ (}\mu\text{m)}$	2.21 ± 0.07	2.36 ± 0.02	2.36 ± 0.01
$d_{90} \text{ (}\mu\text{m)}$	4.48 ± 0.19	5.43 ± 0.29	5.37 ± 0.29
Span	1.57 ± 0.04	1.91 ± 0.13	1.91 ± 0.16

Mean $\pm$ standard deviation, n=3

d_{10} - volume diameter under which 10% of the sample resides.

d_{50} - volume median diameter

d_{90} - volume diameter under which 90% of the sample resides.

Span = $(d_{90} - d_{10})/d_{50}$

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3.3.3. Powder crystallinity

The diffractogram of the raw materials, single spray-dried powders and co-spray-dried powder is shown in Figure 3.2. The sharp peaks found in the diffractograms of Raw-CIP and Raw-BP indicated that both starting materials were crystalline powders. The XRD pattern of SD-BP showed only a single diffused broad peak, indicating its amorphicity. In comparison, reduced crystallinity was observed for SD-CIP. This observation was not surprising as we have previously reported on the reduced crystallinity of SD-CIP on the Nano Spray Dryer B90 [37]. The XRD pattern of SD-BP/CIP appeared to be very similar to that of SD-CIP due to the high weight ratio of CIP in the formulation.

Figure 3.2. XRD patterns of Raw-CIP, Raw-BP, SD-BP, SD-CIP and SD-BP/CIP. Reproduced with permission from Ref. [27]. Copyright 2014 Elsevier

3.3.4. Surface composition evaluation

The XPS technique was used to detect the chemical composition of the particle surface and to determine the drug distribution across the surface. The surface composition evaluation by XPS demonstrated an enrichment of hydrophobic BP on the combination particle surfaces, which could contribute to the reduction of particle cohesiveness and hence the improvement of SD-BP/CIP dispersibility. Table 3.3. illustrates the experimentally measured surface composition of the raw materials and spray-dried powders that was derived from the XPS peak areas. Generally, the experimentally measured surface elemental composition of the raw materials (i.e. Raw-BP and Raw-CIP) was comparable to their spray-dried counterparts (i.e. SD-BP and SD-CIP). This implies that the possibility of surface composition alteration via spray-drying process was minimal. On the other hand, the experimentally measured surface

composition of the binary spray-dried powder, SD-BP/CIP, did not follow the trends of either SD-BP or SD-CIP. Hence, neither BP nor CIP solely resided on the surface of SD-BP/CIP. In order to further evaluate the surface composition of binary SD-BP/CIP, the expected elemental weight percentages ($Wt_{\text{expected}} \%$) of the binary SD-BP/CIP was calculated (by assuming that both BP and CIP were distributed evenly on the particle surface). The expected elemental weight percentages could be calculated from the elemental weight percentages that were measured experimentally on the raw materials (Table 3.4.). For example, the expected elemental weight percentage of carbon in SD-BP/CIP was the summation of the products obtained via multiplying the $Wt.\%$ of carbon in Raw-BP with weight proportion of BP (i.e. 3% w/w) in SD-BP/CIP and $Wt.\%$ of carbon in Raw-CIP with the weight proportion of CIP in SD-BP/CIP (i.e. 97% w/w). The experimentally measured elemental weight percentages ($Wt. \%$) of the binary SD-BP/CIP were then compared with the expected elemental weight percentages ($Wt_{\text{expected}} \%$). Any differences between the $Wt. \%$ and $Wt_{\text{expected}} \%$ suggested an over- or under-abundance of certain elements and hence the types of molecules on the particle surface. From Table 3.4., the $Wt_{\text{expected}} \%$ of the binary SD-BP/CIP was comparable to the $Wt.\%$ of Raw-CIP and SD-CIP. It was as expected for an assumed even distribution of BP and CIP on the particle surface. BP being a very minor component (just 3% weight ratio), would be considered negligible when compared to the presence of CIP (97% weight ratio) on the surface.

Nitrogen and fluorine are two elements that are specific to CIP ($C_{17}H_{18}FN_3O_3.HCl$) but not BP ($C_{28}H_{37}ClO_7$), so a lower C/N, C/F, O/N, O/F, Cl/N and Cl/F experimental ratio in SD-BP/CIP over that of SD-CIP could

possibly suggest that CIP resided less preferentially on the surface of SD-BP/CIP as compared to BP (Table 3.5.). However, due to the large weight ratio of CIP (97%) in SD-BP/CIP, some of the CIP molecules could still be found on the particle surface of SD-BP/CIP. The enrichment of BP on the particle surface could also be inferred from the C/O ratios. SD-BP has a higher C/O ratio than SD-CIP and the high C/O ratio obtained for SD-BP/CIP again implies that BP preferentially resides on the surface of the particle.

The enrichment of BP on the particle surface is an expected outcome, as during spray drying, the more volatile solvent (i.e. methanol) in a mixed methanol-water droplet would evaporate much faster than water. Due to the low solubility of BP in the remaining aqueous phase, precipitation of the compound onto the surface of the alcohol-deficient droplets and hence the dried particle, is hence encouraged. Similarly, Kumon et. al. [45] had previously shown that hydrophobic drugs (rather than the hydrophilic excipient) tend to reside on the particle surface when spray-dried from an alcohol-water mix.

Table 3.3. Surface composition of the raw materials and spray-dried powders^a

Sample	Element	At. %	Wt. %
Raw BP (C ₂₈ H ₃₇ ClO ₇)	Carbon	73.9	60.5
	Oxygen	17.9	19.6
	Chlorine	8.2	19.9
Raw CIP (C ₁₇ H ₁₈ FN ₃ O ₃ .HCl)	Carbon	63.9	49.7
	Oxygen	11.3	11.8
	Chlorine	10.7	24.7
	Nitrogen	10.7	9.7
	Fluorine	3.4	4.1
SD-BP (C ₂₈ H ₃₇ ClO ₇)	Carbon	72.4	59.7
	Oxygen	20.1	22.1
	Chlorine	7.5	18.2
SD-CIP (C ₁₇ H ₁₈ FN ₃ O ₃ .HCl)	Carbon	64.0	52.1
	Oxygen	12.6	13.5
	Chlorine	8.6	20.3
	Nitrogen	10.5	9.8
	Fluorine	3.4	4.3
SD-BP/CIP (C ₂₈ H ₃₇ ClO ₇) (C ₁₇ H ₁₈ FN ₃ O ₃ .HCl)	Carbon	53.6	42.4
	Oxygen	35.6	37.5
	Chlorine	7.0	16.3
	Nitrogen	3.0	2.8
	Fluorine	0.9	1.1

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Table 3.4. Calculation of expected elemental percentage in SD-BP/CIP^c

Sample	Drug component	Wt. proportion (%) ^a	Theoretical elemental Wt.% ^b		Expected elemental Wt.% ^c		Experimental elemental Wt.% ^d	
SD-BP/CIP	BP	3	Carbon	1.8	Carbon	50.0	Carbon	42.4
			Oxygen	0.6	Oxygen	12.0	Oxygen	37.5
			Chlorine	0.6	Nitrogen	9.4	Nitrogen	2.7
					Chlorine	24.6	Chlorine	16.3
	CIP	97	Carbon	48.2	Fluorine	4.0	Fluorine	1.1
			Oxygen	11.4	Total	100	Total	100
			Nitrogen	9.4				
			Chlorine	24.0				
			Fluorine	4.0				

^aWeight proportion (%) = [weight ratio of BP or CIP in feed/(total weight ratio in feed)]x100; weight ratio of BP:CIP in feed = 1:32.5

^bTheoretical elemental Wt.% = Wt.% (raw materials) x weight proportion

^cExpected elemental Wt.% = summation of theoretical elemental Wt. % (e.g for carbon, expected elemental Wt.% = 1.8 + 48.2 = 50.0); assuming both BP and CIP were distributed evenly on particle surface

^dExperimental elemental Wt.% = At.% x atomic weight of the element

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Table 3.5. Various elemental ratios of the spray-dried powders^a

Ratio	SD-BP	SD-CIP	SD-BP/CIP
Carbon/oxygen	1:0.37	1:0.26	1:0.88
Carbon/nitrogen	-	1:0.19	1:0.07
Carbon/fluorine	-	1:0.08	1:0.03
Oxygen/nitrogen	-	1:0.73	1:0.07
Oxygen/fluorine	-	1:0.32	1:0.03
Chlorine/nitrogen	-	1:0.48	1:0.17
Chlorine/fluorine	-	1:0.21	1:0.07

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3.3.5. In vitro aerosol performance

In this study, the aerosol performance of the spray-dried powders was studied under two levels of realistic inspiratory effort: low (60 L/min) and high (100 L/min). The lower air flow of 60 L/min is likely to be generated by patients with compromised lung functions (e.g. pneumonia [37]) while the higher air flow of 100 L/min through the Aerolizer[®] inhaler represents a comfortable inspiratory effort readily achieved by patients with healthy lung functions [45, 48, 49].

The aerosol performance results of the spray-dried powders are shown in Figure 3.3. and Table 3.6. Generally, SD-CIP was found to perform poorer, with FPFs of $17.8 \pm 1.0\%$ at 60L/min and $25.1 \pm 4.0\%$ at 100 L/min as compared to SD-BP which had FPFs of $32.0 \pm 1.2\%$ at 60 L/min and $41.7 \pm 0.3\%$ at 100 L/min. This might be due to SD-CIP's higher agglomeration propensity in the aerosol (evident via the high powder deposition throughput on Stage 1) (Figure 3.3b). However, agglomerate break-up was achievable when the flow rate was

increased from 60 to 100 L/min (Figure 3.3e). For SD-CIP, powder deposition on Stage 1 decreased from $24.0 \pm 0.2\%$ to $19.5 \pm 0.1\%$ with an increase in flow rate. In addition, increasing the flow rate from 60 to 100 L/min also has the advantage of improving the emitted fraction (i.e. inferred via an improved FPF but similar FPF(emitted)) (Table 3.6.).

Interestingly, the MSLI deposition profile of binary SD-BP/CIP followed that of SD-BP rather than SD-CIP, even though SD-CIP was the major component in the formulation (wt. ratio of 1:32). Possibly, this could be due to the distribution of BP on the particle surface of SD-BP/CIP, which in a way reinforces the earlier findings from the XPS and SEM analyses. For the binary SD-BP/CIP, there was concomitant deposition observed across all stages (including the throat, inhaler and capsule) at 60 and 100 L/min, suggesting uniformity in the powder and/or aerosol (Figure 3.3c & 3.3f). From Table 3.6., it also does seem that formulating the drugs into a binary mix has the advantage of improving their delivery more effectively to the patient. FPF and FPF (emitted) for SD-BP and SD-CIP were generally much lower than BP and CIP in SD-BP/CIP. For CIP in particular, the improvement was significant when it was co-delivered with BP at 60 and 100 L/min.

Table 3.6. Deposition parameters (mean $\pm$ standard deviation, n = 3) of different formulations measured by MSLI at 60 and 100 L/min^c

	60L/min				100L/min			
	SD-BP	SD-CIP	SD-BP/CIP		SD-BP	SD-CIP	SD-BP/CIP	
			BP	CIP			BP	CIP
FPF ^a (%)	32.0 $\pm$ 1.2	17.8 $\pm$ 1.0	36.4 $\pm$ 1.3	38.3 $\pm$ 0.8	41.7 $\pm$ 0.3	25.1 $\pm$ 4.0	44.5 $\pm$ 2.1	45.9 $\pm$ 1.5
FPF (emitted) ^b (%)	47.6 $\pm$ 1.2	21.6 $\pm$ 2.0	49.0 $\pm$ 2.5	51.3 $\pm$ 1.9	48.5 $\pm$ 0.2	33.8 $\pm$ 3.7	50.0 $\pm$ 0.7	50.6 $\pm$ 0.2

^a FPF - fine particle fraction

^b FPF (emitted) - emitted fine particle fraction

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Figure 3.3. In vitro deposition of (a) SD-BP, (b) SD-CIP, (c) SD-BP/CIP at 60 L/min and (d) SD-BP, (e) SD-CIP and (f) SD-BP/CIP at 100 L/min. Data presented as mean $\pm$ standard deviation (n=3). S1-S4 denote impactor stages, followed by their corresponding lower aerodynamic cut-off diameter in parentheses. Reproduced with permission from Ref. [27]. Copyright 2014 Elsevier

3.3.6. In vitro drug release

BP, being a Biopharmaceutical Classification System (BCS) class II drug (i.e. low solubility, high permeability) is limited by dissolution-rate limited kinetics [50]. Therefore, any improvements to the drug's pulmonary solubility would be beneficial to the absorption process in the lungs [51].

Figure 3.4. shows that when BP (a poorly water soluble drug) was formulated into a binary mix with the water soluble CIP, its dissolution rate and extent were drastically improved over the single species. Hence, this formulation approach does seem to be highly effective in 'lifting' the performance of the poorer-performing species to almost the same extent as the robust species. Drug release of CIP in the binary mix was comparable to that of the single species. The release mechanism in this case was analogous to an 'API-API solid dispersion system' whereby the poorly water-soluble BP was dispersed into the more water-soluble CIP matrix, hence enhancing the wettability and solubility of the former (Figure 3.5.) [52].

Figure 3.4. In vitro dissolution rate profiles of CIP and BP in individual and combined formulations. Reproduced with permission from Ref. [27]. Copyright 2014 Elsevier

Figure 3.5. Schematic diagram illustrating release mechanism. Reproduced with permission from Ref. [27]. Copyright 2014 Elsevier

3.3.7. Antimicrobial activity of spray-dried powders

Klebsiella pneumoniae, *Pseudomonas aeruginosa* and *Staphylococcus aureus* are common etiologic agents for CAP [4]. Table 3.7. shows the antibacterial activity of the spray-dried powders with MICs of SD-BP, SD-CIP and SD-BP/CIP determined via the microdilution method on type strains of *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. As expected, the MIC of SD-BP was not determinable as BP is a typical corticosteroid that does not exhibit any antibacterial properties. The MICs of SD-BP/CIP on the various bacterial species were found to be comparable to the MICs of SD-CIP, suggesting the antibacterial properties of CIP in the binary mix was adequately retained.

Table 3.7. MIC values of the spray-dried powders^b

Bacteria species and strain	MIC (µg/ml)		
	SD-BP	SD-CIP	SD-BP/CIP
<i>Klebsiella pneumoniae</i> ATCC 12885	ND ^a	1 - 2	2
<i>Pseudomonas aeruginosa</i> ATCC 90207	ND	2	1
<i>Staphylococcus aureus</i> ATCC 4330	ND	1 - 2	2

^a ND - not determined due to the absence of inhibition

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3.4. Conclusions

A novel steroid-antibiotic DPI formulation (SD-CIP/BP) was successfully prepared via nanospray drying. The powder obtained was of respirable-size (d_{50} of $\sim 2.3 \mu\text{m}$), partially crystalline (potentially improved storage stability) and had BP preferentially deposited on the particle surfaces. When dispersed via an Aerolizer[®] at 60 L/min and 100 L/min, the powder showed concomitant and uniform *in vitro* deposition profile across all impaction stages, with obtained FPFs of $\sim 36\text{-}46\%$. Favourably, when formulated as a binary mix, both CIP and BP showed much higher drug release and FPFs over their singly-delivered counterparts, and was also active against the respiratory tract infection-causing bacteria *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Co-spray-dried powder of BP and CIP (SD-BP/CIP) may hence be a novel and useful anti-inflammatory and antimicrobial formulation for CAP patients with COPD complications.

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Chapter 4

A Novel Inhaled Multi-Pronged Attack against Respiratory Bacteria

4.1. Introduction

Airway mucus hypersecretion and a recalcitrant bacterial infection are the hallmarks of cystic fibrosis (CF), and as of today, this condition is the most common life-threatening inherited disease afflicting the Caucasian population [1]. The CF disorder is caused by mutations in a single gene on chromosome 7 that encodes the cystic fibrosis transmembrane conductance regulator (CFTR), a 1480 amino acid polypeptide which regulates the transport of chloride ions across epithelial membranes [2]. Abnormalities of CFTR function result in low volume abnormal secretions leading to mucus plugs, impaired mucociliary clearance and eventually, chronic lung inflammation and infection [3].

As CF patients with *Pseudomonas aeruginosa* infections typically have more rapid declines in lung function than non-*Pseudomonas aeruginosa* infected individuals [3], anti-pseudomonal therapy for this group of patients should hence be initiated early and aggressively upon first isolation of the bacteria, in order to prevent its evolution into a chronic infection [4]. Early eradication of *Pseudomonas aeruginosa* infection is extremely crucial as initial *Pseudomonas aeruginosa* colonization typically involves only non-mucoid strains that are highly susceptible to antibiotic agents. Failure to eradicate this initial colonization would allow the non-mucoid strains to freely mutate into the alginate-coated mucoid strains, which are more resistant to antibiotic agents.

Resistance is brought about as a consequence of poor penetration of the antibiotic agents into the anaerobic sputum plugs [5-7].

Currently, antibiotic therapy remains the mainstay of treatment as gene or protein replacement therapies have yet to become commercial realities [8]. Antibiotic treatment is aimed at reducing the bacterial load and inflammatory response in the lung, so as to ameliorate infective exacerbations and prevent or delay progressive respiratory failure.

Combination drugs, as innovations in pharmacotherapy [9], are increasingly used to combat the spread of antibiotic-resistant bacterial pathogens [9-11]. A combination of antibiotics provides a much broader spectrum of coverage than any single antibiotic alone, as the probability of a bacterium developing resistance to all the antibiotics employed in the combination therapy is much lower than in the case for an antibiotic in monotherapy [12]. Hence, this treatment approach is considered useful in the prevention and treatment of resistant bacterial infections [11, 13-16], and whenever a favourable synergistic effect is achieved, the whole treatment regimen thereby becomes more valuable, as the same killing effect could be obtained at much reduced doses and with fewer side effects [13]. Although antibiotic combinations are commonly engineered in medicine to broaden the antimicrobial spectrum and generate synergistic effects, combinations of antibiotics with non-antibiotic drugs are increasingly gaining popularity as well [17-20]. These non-antibiotic drugs could potentially augment the activity of the antibiotic/s to bring about a more favourable outcome to the patient.

Mucus hypersecretion (increased mucus secretion) is a hallmark of CF whereby the rate of mucus secretion far exceeds the rate of removal by normal ciliary

action [21]. The accumulation of thickened mucus secretions in the lower airway of patients with CF provides a nutrient-rich breeding ground for bacteria that promotes their growth and limits the ease of effective eradication.

To ensure maximum kill and effective penetration of antibiotics through the thick mucus, a novel multi-pronged strategy involving a combined synergy, quorum quenching and mucociliary clearance approach is proposed. Quorum sensing is the mechanism employed by pathogenic bacteria to mount successful infections by allowing them to regulate expression of gene networks that coordinate virulence, antibiotic resistance and fitness responses. The process is mediated by small diffusible signaling molecules [22]. Interference of the quorum sensing system by quorum quenching is recognized as a potential strategy for disease control [23, 24]. Hence, in this study, antibiotic synergism (i.e. combined activity is more than the sum of individual activities) is expected to enhance the bactericidal activity [10, 25], quorum quenching to disrupt bacterial cell-to-cell communication essential for infection and survival [24], and mucociliary clearance to remove/reduce the mucus layer for effective targeting of underlying bacteria [21]. As airway mucus hypersecretion is a common clinical feature of many severe respiratory diseases like asthma, chronic obstructive pulmonary disease (COPD) and CF [21, 26], the proposed multi-pronged treatment strategy is thus expected to be highly applicable and beneficial to a diverse group of patients (e.g. pneumonia-COPD patients) [20].

Most of the material in this chapter was published in *European Journal of Pharmaceutical Sciences* [27]. Some modifications to the text are included here.

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4.2. Aim

The aim of this work is to explore a useful inhaled antibiotic-(non-antibiotic) formulation that comprises the use of a mucoactive agent with the antibiotic/s to facilitate simultaneous treatment and clearance of respiratory bacteria and mucus respectively (mucoactive agents are drugs used to alter the viscoelastic properties of mucus and promote secretion clearance [21]). Hence, this work seeks to develop a highly-efficacious ternary dry powder inhaler (DPI) formulation (ciprofloxacin hydrochloride (CIP), gatifloxacin hydrochloride (GAT) and ambroxol hydrochloride (AMB)) capable of delivering a novel multi-pronged attack (Synergy, Quorum quenching and Mucociliary clearance) on *Pseudomonas aeruginosa*, a common respiratory bacteria found in mucoid infections. In this work, CIP and GAT were selected as the synergistic antibiotic pair [25], while ambroxol hydrochloride (AMB) was chosen for its multi-faceted properties [28] that include quorum quenching [24, 29, 30], mucoactivity [28] and pulmonary protective effects [31]. In addition, ambroxol could potentially increase the bioavailability of antibiotics in the lung, particularly in the alveolar region [32]. This effect had been demonstrated for the fluoroquinolones [32] and other drugs [33], and similar benefits may be extended to the antibiotic-mucoactive combination.

4.3. Results and discussion

4.3.1. Surface morphology and particle size

The surface morphologies of the raw materials and spray-dried powders are shown in Figure 4.1. While the Raw-CIP, Raw-GAT and Raw-AMB particles appeared as irregularly-shaped chips and chunks, the spray-dried particles were

generally spherical in geometry. For the spray-dried combination particles, SD-CIP/GAT/AMB/LEU (Figure 4.1h) had a slightly corrugated surface, whereas SD-CIP/GAT/AMB (Figure 4.1g) was smoother in surface morphology. This difference could be due to the presence of the anti-adherent, leucine, which altered the surface properties of the former [34]. Process yields for the various formulations are approximately 50-60% and the particle size distribution of the formulations is summarized in Table 4.1. The particle size analysis revealed that all the spray-dried powders had d_{50} ranging from 1.11 ± 0.05 to 2.08 ± 0.07 μm .

Figure 4.1. FESEM images of raw materials and spray-dried powders. (a) Raw-CIP, (b) Raw-GAT, (c) Raw-AMB, (d) SD-CIP, (e) SD-GAT, (f) SD-AMB, (g) SD-CIP/GAT/AMB and (h) SD-CIP/GAT/AMB/LEU. Reproduced with permission from Ref. [27]. Copyright 2015 Elsevier

Table 4.1. Volume particle size distribution of spray dried powder^a

	SD-CIP	SD-GAT	SD-AMB	SD-CIP/GAT/AMB	SD-CIP/GAT/AMB/LEU
d ₁₀ (μm)	1.03 ± 0.02	1.10 ± 0.01	0.59 ± 0.05	0.54 ± 0.05	0.76 ± 0.02
d ₅₀ (μm)	2.02 ± 0.04	2.08 ± 0.07	1.41 ± 0.05	1.11 ± 0.05	1.61 ± 0.03
d ₉₀ (μm)	3.99 ± 0.24	4.00 ± 0.19	3.09 ± 0.10	2.15 ± 0.05	3.26 ± 0.13
Span	1.46 ± 0.11	1.39 ± 0.05	1.79 ± 0.14	1.45 ± 0.15	1.55 ± 0.07

Mean ± standard deviation, n=3

d₁₀ - volume diameter under which 10% of the sample resides

d₅₀ - volume median diameter

d₉₀ - volume diameter under which 90% of the sample resides

Span = (d₉₀-d₁₀)/d₅₀

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4.3.2. Powder crystallinity

The diffractogram of the raw materials, single spray-dried and cospray-dried powders is shown in Figure 4.2. The sharp peaks found in the diffractograms of Raw-CIP, Raw-GAT and Raw-AMB indicated that the starting materials were crystalline powders. While SD-CIP and SD-GAT powders were largely amorphous, SD-AMB exhibited reduced crystallinity. SD-CIP/GAT/AMB and SD-CIP/GAT/AMB/LEU powders were amorphous.

Figure 4.2. XRD patterns of Raw-CIP, Raw-GAT, Raw-AMB, SD-CIP, SD-GAT, SD-AMB, SD-CIP/GAT/AMB and SD-CIP/GAT/AMB/LEU. Reproduced with permission from Ref. [27]. Copyright 2015 Elsevier

4.3.3. In vitro aerosol performance

The aerosolization performance results of the spray-dried powders are illustrated in Figure 4.3. and Table 4.2. In this work, airflow of 60 L/min was used to simulate the reduced inspiratory effort achieved by patients with compromised lung functions (e.g. cystic fibrosis and pneumonia) [20, 25].

Generally, when the spray-dried powders were delivered individually (as single component systems), their FPFs ranged from being poor to marginally acceptable. In particular, SD-AMB exhibited a very low FPF of $8.6 \pm 0.1\%$, which was not surprising, as AMB is known to be highly hygroscopic and cohesive (an FPF value that is significantly higher than 30% is an index of good aerosol performance [25, 35]). Device retention for all three compounds was substantial (i.e. $\approx 40\text{-}60\%$) which also reflected on the cohesiveness of the individual spray-dried powders. Favorably, when formulated as a ternary mix, this retention decreased to under 30% (SD-CIP/GAT/AMB, Figure 4.3d), and with further addition of the anti-adherent LEU, the retention amount dipped to under 15% (SD-CIP/GAT/AMB/LEU, Figure 4.3e). Capsule retention for the latter was also found to be negligible (Figure 4.3e). Generally, for the combination particles which had become single entities after co-spray drying, concomitant deposition was observed across the stages (including the throat, inhaler and capsule), with the exception of the lower stages (stages 3 to filter), where particle breakup probably contributed to the slight deviation in concomitancy. A possible explanation might be due to the impaction of the combination particles at the throat and the early stages of the MSLI that resulted in the release of particle fragments which were then captured downstream. These particle fragments might contain a higher proportion of one drug over the others, thus resulting in the slight deviation in concomitancy for the downstream stages.

From Table 4.2., it can be seen that the FPF of AMB could be improved when it was formulated as a ternary mix with the other drugs and excipient. For SD-CIP/GAT/AMB, the FPF was lifted from $8.6 \pm 0.1\%$ to $14.7 \pm 0.0\%$, and in the presence of LEU (i.e. SD-CIP/GAT/AMB/LEU), it improved significantly to

$64.7 \pm 2.5\%$. Favorably, for the optimized formulation with leucine, the FPF of CIP, GAT and AMB were lifted from low-to-modest values of $24.1 \pm 0.5\%$, $17.2 \pm 0.6\%$ and $8.6 \pm 0.1\%$ respectively in the individuals, to more uniform and optimal values of $69.2 \pm 0.4\%$, $65.8 \pm 0.6\%$ and $64.7 \pm 2.5\%$ respectively. Furthermore, there was also an improvement to the emitted fraction (i.e. inferred via closely similar FPF and FPF (emitted)). Therefore, co-delivery does seem to be highly beneficial in improving the dispersibility of the actives. SD-CIP/GAT/AMB/LEU was aerodynamically more superior than SD-CIP/GAT/AMB as the anti-adherent LEU had the beneficial effect of altering the surface properties of the particles [34] to afford a slightly collapsed/corrugated spray-dried morphology, which favorably helped to improve the dispersive behavior (Figure 4.1h) [36, 37]. Indeed, particle morphology has long been known to be an important formulation factor influencing robust aerosol deposition [38, 39]. In contrast to SD-CIP/GAT/AMB and the single component systems, the MMAD of SD-CIP/GAT/AMB/LEU was of the same order of magnitude as its d_{50} , and within the 1-5 μm aerodynamic diameter range, thus indicating its robustness in aerodynamic performance (Table 4.2.). The MMAD values of SD-CIP/GAT/AMB and of the single component systems were much higher than their d_{50} , due to their higher propensity for particle aggregation (formulations lacking anti-adherent LEU) which resulted in much ‘larger particles’ which were mainly captured at the upstream stages (e.g. capsule, device, throat, stage 1). As the GSD, span and d_{50} were similar for SD-CIP/GAT/AMB/LEU and SD-CIP/GAT/AMB, the improvements to the aerodynamic qualities of the former could potentially be attributed mainly to the change in morphology rather than particle size.

Figure 4.3. In vitro deposition of (a) SD-CIP, (b) SD-GAT, (c) SD-AMB (d) SD-CIP/GAT/AMB and (e) SD-CIP/GAT/AMB/LEU at 60 L/min. Data presented as mean $\pm$ standard deviation (n=3). S1-S4 denote impactor stages, followed by their corresponding aerodynamic cut-off diameter in parentheses. Reproduced with permission from Ref. [27]. Copyright 2015 Elsevier

Table 4.2. Deposition parameters of different formulations measured by MSLI at 60 L/min^e

	SD-CIP	SD-GAT	SD-AMB	SD-CIP/GAT/AMB			SD-CIP/GAT/AMB/LEU		
				CIP	GAT	AMB	CIP	GAT	AMB
FPF ^a (%)	24.1 ± 0.5	17.2 ± 0.6	8.6 ± 0.1	21.2 ± 0.6	16.2 ± 0.1	14.7 ± 0.0	69.2 ± 0.4	65.8 ± 0.6	64.7 ± 2.5
FPF(emitted) ^b (%)	50.3 ± 0.7	53.0 ± 0.4	18.0 ± 0.2	31.5 ± 0.1	24.9 ± 0.7	22.2 ± 0.9	79.8 ± 0.3	76.6 ± 0.1	73.4 ± 0.9
MMAD ^c (μm)	16.8 ± 0.9	28.4 ± 0.4	14.6 ± 0.1	16.5 ± 0.3	20.0 ± 0.2	11.7 ± 1.3	3.1 ± 0.1	3.6 ± 0.1	4.1 ± 0.3
GSD ^d	4.4 ± 0.2	5.3 ± 0.2	1.8 ± 0.0	3.7 ± 0.0	3.6 ± 0.1	1.8 ± 0.2	3.3 ± 0.0	3.1 ± 0.1	2.4 ± 0.1

^aFPF - fine particle fraction

^bFPF(emitted) - emitted fine particle fraction

^cMMAD - mass median aerodynamic diameter

^dGSD - geometric standard deviation

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4.3.4. Antimicrobial activity of spray-dried powders

Figure 4.4a shows the dose response kill curve of SD-CIP/GAT and the optimized SD-CIP/GAT/AMB/LEU compounds in artificial sputum medium (ASM) spiked with *Pseudomonas aeruginosa*. Indeed, with the addition of AMB into the formulation, the bacterial killing effect was improved. The rate of $\log_{10}$ CFU decrease with antibiotic concentration was much faster for SD-CIP/GAT/AMB/LEU than SD-CIP/GAT, which suggested the presence of a ‘barrier-free’ environment that promoted strong antibiotic-bacteria interactions (i.e. breaking down the bacterium’s ‘shield’). For SD-CIP/GAT/AMB/LEU, full eradication of bacteria (i.e. $\log_{10}$ CFU = 0) was achieved at approximately half the antibiotic concentration required for SD-CIP/GAT (i.e. ≈ 0.25 and $0.45 \mu\text{g/ml}$ respectively). This is indeed highly beneficial as it would mean that the effective penetration of antibiotics to the underlying bacteria could potentially result in lesser antibiotics needed for the same kill. This would have positive implications in terms of antibiotic dosage reduction, minimization of toxic side effects and the risk of encountering antibiotic resistance (due to the excessive use of valuable antibiotics). At present, the use (overuse, misuse and underuse) of antibiotics is driving the emergence of antimicrobial resistance in bacterial pathogens [40, 41]. Therefore, improved killing at much lower drug doses, coupled with the ‘portability’ and ‘inhaled’ nature of the formulation (i.e. dry powder inhalers) could potentially improve treatment outcomes and accelerate patient recoveries.

The use of AMB in the formulation has benefits in terms of (1) destroying the structure of ASM [29, 42], (2) decreasing the bacterial load (via quorum quenching) and (3) allowing effective penetration of the synergistic antibiotics into the underlying bacteria in mucoid infections. AMB has significant

antagonistic quorum-sensing properties [29] and in combination with the antibiotics, results in the ability to reduce bacterial cell counts more effectively via enabling drug penetration and destruction of the biofilm-caged bacteria. As expected, SD-AMB/LEU as a negative control (i.e. non-antibiotic) showed no bactericidal activities (Figure 4.4b).

Figure 4.4. Dose response kill curve of SD-CIP/GAT/AMB/LEU compared to (a) SD-CIP/GAT and (b) SD-AMB/LEU (control) in artificial sputum medium (ASM) spiked with *Pseudomonas aeruginosa*. Reproduced with permission from Ref. [27]. Copyright 2015 Elsevier

4.4. Conclusions

A novel ternary antibiotic-(non-antibiotic) DPI formulation was successfully prepared and optimized for mucoid infections. The spray-dried powder particles were of respirable-size (d_{50} of $\sim 1.61 \pm 0.03 \mu\text{m}$) and slightly corrugated. When dispersed via an Aerolizer[®] inhaler at 60L/min, the powder showed concomitant *in vitro* deposition with obtained FPFs of $\sim 64\text{-}69\%$. Favourably, when formulated as a ternary mix, CIP, GAT and AMB showed much higher and uniform FPFs over their singly-delivered counterparts. Tests on ASM spiked with *Pseudomonas aeruginosa* showed that the optimized ternary formulation (with AMB) was more effective at killing the bacteria (i.e. faster rate of killing) than just the synergistic antibiotics alone (binary formulation; without AMB). The advent of AMB into a synergistic formulation has indeed given the formulator the opportunity to mount a highly beneficial multi-pronged attack on respiratory bacteria, in essence: ‘Penetrate’ (mucociliary clearance), ‘Divide’ (quorum quenching) and ‘Conquer’ (synergistic antibiotics)!

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Chapter 5

Tailored Antibiotic Combination Powders for Inhaled Rotational Antibiotic Therapy

5.1. Introduction

The increasing occurrence of multidrug-resistant (MDR) bacteria, or otherwise known as the ‘superbugs’, is an alarming public health-care threat. Of particular concern are the carbapenem (β -lactam class of antibiotics) resistant gram-negative bacteria which acquire resistance through the enzymatic hydrolysis of carbapenems via carbapenemases [1-3]. Infections involving these multidrug-resistant bacteria are often dire in terms of therapeutic options due to the lack of antibiotics that are active against these pathogens. At present, respiratory lung infections due to MDR bacterial pathogens or ‘superbugs’ have very grim clinical outcomes [4] with mortality rates as high as 80% for nosocomial and community-acquired pneumonia [5, 6]. Hence, the development of alternative treatment regimens is a pressing need.

Colistin (also known as polymyxin E) is a 50-year old cationic polypeptide antibiotic that is experiencing clinical revival. During the early years of its clinical use, nephrotoxicity and neurotoxicity [7] issues diminished its popularity. Its current resurgence as a ‘last line’ therapeutic against hard-to-treat MDR respiratory pathogens such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumoniae* [8], arises as many of these organisms still retain susceptibility towards it.

However, in recent years, sporadic worldwide reports of colistin-resistance have emerged (e.g. United States [3], Singapore [9], Greece [10, 11], United

Kingdom [12] and South Korea [13]) [3]. Therefore, in an effort to extend the therapeutic life of colistin, combination regimens based on colistin as the ‘anchor’ drug as opposed to monotherapy, is proposed as a strategy for combating drug resistance. Combinatorial therapy has several benefits. Firstly, from the microbiological standpoint, it thwarts the emergence of bacterial drug resistance as the simultaneous development of resistance towards multiple drug classes is remote [14]; and secondly from a patient management standpoint, it has the potential to reduce colistin toxicity by allowing a lowered therapeutic dose. This is achieved via a synergistic or additive interaction of colistin with its partner antibiotics [8, 15, 16].

When route of administration of antibiotics is considered, inhaled delivery of colistin combinations is more effective in eradicating MDR pathogens as it directly targets the foci of infection. Elevated drug concentrations can be achieved locally at the site of lung infection without the adverse effects of systemic toxicity [17]. A dry powder inhaler (DPI) formulation is superior over the other aerosol delivery modes (e.g. nebuliser or metered dose inhaler (MDI)) in terms of stability, delivery efficiency, portability, ease-of-use and the avoidance of undesired precipitation in solutions [18-21].

To date, to the best of the authors’ knowledge, there has been no commercially-available inhaled DPI product for the eradication of respiratory ‘superbugs’. In addition, no DPI combination involving a synergistic ternary mechanism of action on resistant bacteria (with actual successful testing on resistant species) has ever been reported in the academic literature (previous efforts at best were on binary synergism and tested on non-resistant strains [15]). Commercially, the only antibiotic DPI therapies that are currently available or in the pipeline,

have been limited to just the single species (i.e. tobramycin, colistin and ciprofloxacin monotherapies) [15, 21-24] and these are clearly not indicated for the specific treatment of MDR pathogens.

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5.2. Aim

In this work, we thereby sought to identify, formulate and then evaluate the performance of three ternary colistin DPI combinations (i.e. colistin/tigecycline/rifampicin (COL/TIG/RIF); colistin/meropenem/rifampicin (COL/MEM/RIF); colistin/meropenem/tigecycline (COL/MEM/TIG)) that could potentially be useful in the fight against respiratory ‘superbugs’. Colistin was the ‘anchor’ drug, while the other two species were chosen from distinct antibiotic classes (conferring distinct killing mechanisms) to make up the ‘multiple-classed’ ternary cocktails (i.e. three derived permutations).

5.3. Results and discussion

5.3.1. Identification of effective ternary colistin combinations

Combination therapy, in particular, synergistic (the combined effect is greater than the sum of the individual effects) and additive (the combined effect is the sum of the individual effects) formulations, are important innovations in pharmacotherapy [27], in the drought of newer antibiotic discoveries to counter

the growing threat of antimicrobial resistance. However, identification of suitable candidates at efficacious proportions is crucial and not as straightforward as it seems, as incompatible combinations could lead to antagonistic interactions that might be harmful or even fatal to the patients [28].

Due to decreased antibiotic development effort within the pharmaceutical industry, colistin is nowadays increasingly and widely recommended as the last-resort therapeutic option against infections caused by MDR Gram-negative pathogens [8]. In this work, colistin was therefore the anchor drug around (with meropenem, rifampicin or tigecycline as auxiliaries) which the different colistin combinations (Table 5.1.) were developed and formulated into ternary aerosol dry powders. Meropenem, rifampicin and tigecycline are among the antibiotics that have clinically proven track records for the treatment of nosocomial infections [29-34]. Rifampicin is a bactericidal antibiotic drug of the rifamycin group that has been used to prevent and treat tuberculosis and other infections, but has very limited use in monotherapy due to the rapid emergence of resistance [35]. Only in combination with other antimicrobial agents, does rifampicin unleash its innate potential in the treatment of serious infections. Meropenem is a member of the carbapenem class of antibiotic that is active against a broad spectrum of Gram-positive and Gram-negative pathogens, including the beta-lactamase producers [36]. However, the worldwide spread of drug resistance to carbapenem antibiotics [37-39] is seriously threatening this class of life-saving drugs. Though its use in monotherapy has been undermined, the potential application of this antibiotic in combination with other antibiotics to achieve a favourable interaction (e.g. synergism) is still an untapped valuable resource. Tigecycline, the first in a new class of antibiotics called the glycylcyclines, had been approved by the Food and Drug Administration (FDA)

in 2009 for the treatment of community-acquired pneumonia, and was considered a useful alternative against carbapenem-resistant bacteria [40]. However, its clinical efficacy, especially in severe infections, is a cause of concern, and may be due to its subdued bacteriostatic (inhibiting the growth) action (Table 5.1.), low serum levels and the heightened propensity for resistance development (especially during monotherapy) [40, 41]. Although the use of tigecycline in monotherapy does have its limitations, its application in combination therapy is still an unexplored promising opportunity, as the drug could potentially be used to augment the therapeutic properties of the other antibiotic/s in the combination (e.g. as in synergism). Table 5.2. shows experimentally that meropenem, tigecycline and rifampicin are clearly not suited for monotherapy against the resistant strains (i.e. heightened MICs). Therefore, applying them to augment the properties of a primary antibiotic (as in combination therapy) is indeed a useful approach.

Table 5.1. Antimicrobial mechanisms of action and the respective colistin-anchor combination permutations^a

Individual drug	Drug class	Effect on bacteria	Mode of action	Ternary drug combinations (drug ratio by weight %)
Colistin (COL)	Polymyxin	Bactericidal	Disruption of cell membrane	-
Meropenem (MEM)	Carbapenem	Bactericidal	Inhibition of peptidoglycan synthesis	COL/MEM/TIG (5:4:4)
Rifampicin (RIF)	Rifamycin	Bactericidal	Inhibition of RNA transcription	COL/MEM/RIF (5:4:12)
Tigecycline (TIG)	Glycylcycline	Bacteriostatic	Inhibition of protein synthesis	COL/TIG/RIF (5:4:12)

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Table 5.1. shows the different permutations obtained through mixing the ‘anchor’ drug colistin with the other ‘adjuvant’ antibiotics. Each of the antibiotics in the individual combinations is from a different class and hence, would have a different mode of kill on pathogenic bacteria. Figure 5.1. shows a schematic of the antibiotics’ different mechanisms of action. A combination of

antibiotics with a variety of these mechanisms could potentially provide a much broader coverage of kill and prevent the resurgence of superbug resistance. Together with a ‘toolbox’ of three ternary combinations that could possibly be rotated by the physician in ‘rotational therapy’, the possibility of a superbug-infection-related relapse would thus be very far remote.

Figure 5.1. Schematic illustrating the antibiotics’ mechanism of action. Reproduced with permission from Ref. [26]. Copyright 2016 Elsevier

As earlier mentioned, the in vitro susceptibility of control and MDR strains against each individual drug of the different combinations was first determined (Table 5.2.). The MIC values obtained experimentally were further guided by EUCAST (European Committee on Antimicrobial Susceptibility Testing) clinical susceptibility breakpoints [42] (Table 5.2.) to obtain ratios that would

be ‘universally’ potent (Table 5.2.). For colistin, the susceptibility breakpoint for *Pseudomonas* spp. was 4 mg/L, and experimentally, the resistant strains were all sensitive to concentrations under 4 mg/L (Table 5.2.). To further enhance the therapeutic efficiency, the derived MIC for colistin was adjusted to 5 mg/L. For meropenem, while the susceptibility breakpoints for Enterobacteriaceae, *Acinetobacter* spp. and *Pseudomonas* spp. were all at 2 mg/L, it was evident experimentally that it was unsuitable to be applied in monotherapy against resistant *Klebsiella pneumoniae*, *Escherichia coli* and *Acinetobacter baumannii*. As resistant *Pseudomonas aeruginosa* had an MIC of 3 mg/L, the derived MIC for meropenem was therefore adjusted to 4 mg/L to enhance the therapeutic efficiency. For tigecycline, susceptibility breakpoints were not available for *Pseudomonas* spp. and *Acinetobacter* spp., as they were considered fair to poor targets for the drug when applied in monotherapy [42]. Experimentally, tigecycline exhibited a high MIC of 16 mg/L against resistant *Pseudomonas aeruginosa*, thus confirming its monotherapy limitations. While an MIC of under 3 mg/L was sufficient to eradicate resistant *Klebsiella pneumoniae*, *Escherichia coli* and *Acinetobacter baumannii*, experimentally, the derived MIC for tigecycline was adjusted to 4 mg/L to enhance the therapeutic efficiency. For rifampicin, there was unavailability of susceptibility breakpoints, as the drug was not suited for eradicating Enterobacteriaceae, *Acinetobacter* spp. and *Pseudomonas* spp. when applied in monotherapy [42]. This was confirmed by the experimental data on the control and resistant strains, where high MICs were typically needed for effective killing. The derived MIC for rifampicin was based on the experimental MIC values for *Klebsiella pneumoniae* ATCC 13883 and *Acinetobacter baumannii* ATCC 19606 (i.e. 4-12 mg/L). The higher value of 12 mg/L was chosen for an

enhanced therapeutic efficiency. Consequently, the derived MICs of colistin, meropenem, tigecycline and rifampicin were 5 mg/L, 4 mg/L, 4 mg/L and 12 mg/L, respectively. The drug ratios in the combinations were developed from a ratio of the individual drugs' derived MIC values (Table 5.2.).

Table 5.2. Minimum inhibitory concentration (MIC) values (Etest) of colistin, meropenem, tigecycline and rifampicin against various pathogenic respiratory bacteria and their reference EUCAST clinical breakpoints^a

	MIC determination (Etest) (mg/L)			
	COL	MEM	TIG	RIF
Control strains				
<i>Pseudomonas aeruginosa</i> ATCC 90207	1	0.125	8	>32**
<i>Klebsiella pneumoniae</i> ATCC 13883	0.125	0.5	0.125	12
<i>Escherichia coli</i> ATCC 25922	0.125	0.032	0.25	16**
<i>Acinetobacter baumannii</i> ATCC 19606	0.25	0.75	0.5	4
Resistant strains				
<i>Pseudomonas aeruginosa</i> PSE2 (CIP ^R GEN ^R MEM ^R)	1.5	3	16**	>32**
<i>Klebsiella pneumoniae</i> ENT443 (CIP ^R MEM ^R)*	2	>32**	2	>32**
<i>Escherichia coli</i> EC424 (CIP ^R MEM ^R)*	0.5	>32**	1	>32**
<i>Acinetobacter baumannii</i> AB1 (CIP ^R GEN ^R MEM ^R)	1	>32**	2	>32**
EUCAST breakpoint***				
	COL	MEM	TIG	RIF
Enterobacteriaceae	>2	>2	>1	NA
<i>Acinetobacter</i> spp.	>2	>2	IE	NA
<i>Pseudomonas</i> spp.	>4	>2	NA	NA
Derived MICs (Experimental + EUCAST guided) (mg/L)	5	4	4	12
Developed Ratios	COL/MEM/RIF COL/MEM/TIG COL/TIG/RIF			
	5:4:12 5:4:4 5:4:12			

* Isolate bears a carbapenemase which results in enzymatic hydrolysis of carbapenems conferring carbapenem resistance.

** This value suggests that the drug when applied in monotherapy is inadequate for targeting the species.

*** Adapted from http://www.eucast.org/clinical_breakpoints/. Breakpoints indicative of non-susceptibility.

NA: Breakpoints not applicable as the species is considered as a poor target for the drug when applied in monotherapy.

IE: Insufficient evidence that the organism is a good target for therapy with the drug when applied in monotherapy.

EUCAST: European Committee on Antimicrobial Susceptibility Testing

5.3.2. Surface morphology and particle size

The surface morphologies of the single and ternary spray-dried particles are shown in Figure 5.2. The single spray-dried particles (i.e. SD-COL, SD-TIG and SD-MEM), with the exception of SD-RIF, were generally spherical in geometry and had corrugated surfaces. Presence of chips were observed for SD-COL (Figure 5.2a) and SD-TIG (Figure 5.2b), but not for SD-MEM (Figure 5.2c). The presence of these chips may be due to particle break-up during spray drying. Interestingly, the morphology of SD-RIF (Figure 5.2d) closely resembled a crumpled sheet of paper. For the spray-dried ternary combination particles, both SD-COL/TIG/RIF (Figure 5.2f) and SD-COL/MEM/RIF (Figure 5.2g) had crumpled sheet-like structures, whereas SD-COL/MEM/TIG (Figure 5.2e) was a mixture of corrugated spherical particles and irregularly-shaped chips.

Figure 5.2. FESEM images of spray-dried formulations. (a) SD-COL, (b) SD-TIG, (c) SD-MEM, (d) SD-RIF, (e) SD-COL/MEM/TIG, (f) SD-COL/TIG/RIF, and (g) SD-COL/MEM/RIF. Reproduced with permission from Ref. [26]. Copyright 2016 Elsevier

The similarity in surface morphologies of both SD-COL/TIG/RIF and SD-COL/MEM/RIF to SD-RIF may be due to the dominance of rifampicin in the spray-dried ternary combination particles, which resulted in its prevailing influence on their particle morphologies. Process yields for various colistin formulations are approximately 80-90% and Table 5.3. shows the particle size distribution of the spray-dried formulations. The particle size analysis revealed that all the spray-dried formulations were within the respirable size range, with d_{50} of 2.9 ± 0.1 , 1.6 ± 0.1 , 3.2 ± 0.1 , 2.5 ± 0.1 , 3.1 ± 0.3 , 3.4 ± 0.1 and 3.1 ± 0.2 μm for SD-COL, SD-MEM, SD-RIF, SD-TIG, SD-COL/MEM/TIG, SD-COL/MEM/RIF and SD-COL/TIG/RIF, respectively.

Table 5.3. Volume particle size distribution (mean $\pm$ SD, n=3) of spray-dried powders^a

Formulations	d ₁₀ (μm)	d ₅₀ (μm)	d ₉₀ (μm)	Span
SD-COL	1.1 $\pm$ 0.0	2.9 $\pm$ 0.1	8.2 $\pm$ 0.4	2.5 $\pm$ 0.1
SD-MEM	0.9 $\pm$ 0.0	1.6 $\pm$ 0.1	3.0 $\pm$ 0.3	1.3 $\pm$ 0.1
SD-RIF	1.2 $\pm$ 0.0	3.2 $\pm$ 0.1	10.4 $\pm$ 1.1	2.8 $\pm$ 0.2
SD-TIG	1.1 $\pm$ 0.0	2.5 $\pm$ 0.1	6.2 $\pm$ 0.3	2.0 $\pm$ 0.1
SD-COL/MEM/TIG	1.3 $\pm$ 0.3	3.1 $\pm$ 0.3	9.4 $\pm$ 2.1	2.7 $\pm$ 1.0
SD-COL/MEM/RIF	1.2 $\pm$ 0.0	3.4 $\pm$ 0.1	13.5 $\pm$ 1.1	3.6 $\pm$ 0.4
SD-COL/TIG/RIF	1.4 $\pm$ 0.1	3.1 $\pm$ 0.2	7.6 $\pm$ 0.6	2.0 $\pm$ 0.2

d₁₀ - volume diameter under which 10% of the sample residesd₅₀ - volume median diameterd₉₀ - volume diameter under which 90% of the sample residesSpan = (d₉₀-d₁₀)/ d₅₀^aReproduced with permission from Ref. [26]. Copyright 2016 Elsevier

5.3.3. Powder crystallinity

The diffractogram of the raw materials, single and ternary spray-dried powders is shown in Figure 5.3. The sharp and narrow intense diffraction peaks of Raw-MEM and Raw-RIF indicate their highly crystalline nature while the broad and diffuse peaks of Raw-COL and Raw-TIG illustrate their amorphous qualities. After spray-drying, all the ternary combination powders (i.e. SD-COL/MEM/TIG, SD-COL/MEM/RIF and SD-COL/TIG/RIF) presented broad diffused diffraction patterns which were characteristic of amorphous materials. It has been well-reported that spray-dried powders are typically produced as amorphous particles [43].

Figure 5.3. XRD patterns of Raw-COL, Raw-MEM, Raw-RIF, Raw-TIG, SD-COL, SD-MEM, SD-RIF, SD-TIG, SD-COL/MEM/TIG, SD-COL/MEM/RIF and SD-COL/TIG/RIF. Reproduced with permission from Ref. [26]. Copyright 2016 Elsevier

5.3.4. Specific surface area

The specific surface areas of the single and ternary spray-dried formulations are shown in Table 5.4. The specific surface areas of SD-RIF and the ternary spray-dried formulations containing rifampicin (i.e. SD-COL/MEM/RIF and SD-COL/TIG/RIF) were significantly higher than those of the rifampicin-void spray-dried formulations (i.e. SD-COL, SD-MEM, SD-TIG and SD-COL/MEM/TIG). This could be due to the former's crumpled sheet-like particle structure, which minimized aggregation and promoted high specific surface areas [44]. Among the rifampicin-void spray-dried formulations, SD-COL, SD-TIG and SD-COL/MEM/TIG had slightly higher specific surface areas (i.e. 10.34 ± 0.67 to 12.97 ± 0.22 m²/g) than SD-MEM (4.35 ± 0.09 m²/g)

which could probably be explained by the presence of irregularly-shaped chips and hollowed interiors (Figure 5.2.).

Table 5.4. Specific surface areas of spray-dried formulations^a

Formulations	Specific surface area (m ² /g)
SD-COL	12.97 ± 0.22
SD-MEM	4.35 ± 0.09
SD-RIF	51.27 ± 1.71
SD-TIG	10.34 ± 0.67
SD-COL/MEM/TIG	10.80 ± 1.64
SD-COL/MEM/RIF	36.97 ± 0.41
SD-COL/TIG/RIF	47.48 ± 1.30

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5.3.5. Moisture content

Table 5.5. shows the moisture content of the spray-dried powders that were stored at 25°C/40%RH. SD-RIF and SD-TIG exhibited low moisture contents (i.e. 1.0 ± 0.0 and $1.0 \pm 0.1\%$, respectively) which could be due to their lipophilicity [45-48]. In contrast, SD-COL and SD-MEM had higher moisture contents (i.e. 8.0 ± 0.2 and $7.8 \pm 0.3\%$, respectively), as they were hydrophilic drugs [45, 47, 49, 50]. Among the ternary combination formulations, SD-COL/MEM/TIG had much higher moisture content (i.e. $6.8 \pm 0.1\%$) as compared to SD-COL/MEM/RIF ($1.6 \pm 0.0\%$) and SD-COL/TIG/RIF ($1.1 \pm 0.2\%$), and this could be due to the higher proportion of hydrophilic drugs (i.e. colistin and meropenem) in the former. The high proportion of lipophilic species (i.e. mainly rifampicin) in the other two ternary combination formulations (i.e. SD-COL/MEM/RIF and SD-COL/TIG/RIF) could have contributed to their low moisture contents.

Table 5.5. Moisture content of spray-dried powders that were stored at 25°C/40% RH^a

Formulations	Moisture content (%)
SD-COL	8.0 ± 0.2
SD-MEM	7.8 ± 0.3
SD-RIF	1.0 ± 0.0
SD-TIG	1.0 ± 0.1
SD-COL/MEM/TIG	6.8 ± 0.1
SD-COL/MEM/RIF	1.6 ± 0.0
SD-COL/TIG/RIF	1.1 ± 0.2

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5.3.6. Moisture sorption profiles

Moisture sorption isotherms of the single spray-dried formulations (i.e. SD-COL, SD-MEM, SD-RIF and SD-TIG) and ternary spray-dried formulations (i.e. SD-COL/MEM/TIG, SD-COL/MEM/RIF and SD-COL/TIG/RIF) are shown in Figure 5.4a and 5.4b, respectively. For the single species, both SD-MEM and SD-COL exhibited high maximum mass changes of 37% and 34%, respectively, at 90% RH (Figure 5.4a), reflecting the hydrophilicity and high moisture uptake capacity of both materials. SD-MEM and SD-COL were also found to retain some absorbed moisture upon desorption completion, which reflected the materials' irreversible water sorption behaviour. In contrast, the maximum mass changes of SD-TIG and SD-RIF were understandably lower (i.e. 23% and 9%, respectively), as they were lipophilic species. While the water sorption behaviour of SD-TIG was shown to be reversible, the mass loss on desorption exhibited by SD-RIF could have been contributed by the expulsion of residual water [51].

Among the ternary spray-dried formulations (Figure 5.4b), SD-COL/MEM/TIG showed the highest maximum mass change when compared to SD-

COL/MEM/RIF and SD-COL/TIG/RIF. As SD-COL/MEM/TIG contained a high proportion (by weight) of colistin in the formulation, it was hence not surprising to note its maximum mass change to be somewhat similar to that of SD-COL (i.e. ~33%). Favourably, SD-COL/MEM/TIG exhibited reversible water sorption behaviour, in contrast to SD-COL and SD-MEM. Furthermore, no moisture-induced recrystallization was observed for this formulation. SD-COL/MEM/RIF and SD-COL/TIG/RIF had much lower maximum mass changes (i.e. 19% and 13%, respectively), and their sorption curves were somewhat similar from 0 to 60% RH. For SD-COL/MEM/RIF, the formulation was relatively stable and showed no evidence of moisture-induced recrystallization. The mass loss on desorption could have been due to the expulsion of residual water [51]. For SD-COL/TIG/RIF, there was a drop in mass after the humidity reached about 60% RH, which indicated recrystallization and the expulsion of absorbed water [52-54]. Nevertheless, moisture stability for SD-COL/TIG/RIF would not be an issue if it was to be kept under dry conditions (i.e. < 60% RH) (e.g. in moisture-protective blister packs).

Figure 5.4. DVS isotherm of the (a) single spray-dried formulations and (b) ternary spray-dried formulations. Reproduced with permission from Ref. [26]. Copyright 2016 Elsevier

5.3.7. In vitro aerosol performance

The aerosolization performance results of the single and ternary spray-dried formulations are shown in Figure 5.5. and Table 5.6. In this work, airflow of 60 L/min was used to simulate the reduced inspiratory effort achieved by patients with compromised lung functions (e.g. pneumonia and cystic fibrosis) [15, 25]. Generally, all the single and ternary spray-dried formulations displayed robust delivery efficiency with FPFs ranging from $42.6 \pm 1.6\%$ to $69.5 \pm 5.0\%$ (Table 4.7.) (an FPF value that is significantly higher than 30% is an index of good aerosol performance [15, 55]). In particular, among the single spray-dried formulations, SD-MEM was found to have a slightly lower FPF of $42.6 \pm 1.6\%$ when compared to the others (i.e. FPFs > 50%). The lower FPF might be due to the cohesiveness and hygroscopicity of the powder, which could in a way be

inferred by the formulation's high capsule and device retention (i.e. $12.6 \pm 2.8\%$ and $29.0 \pm 2.9\%$, respectively (Figure 5.5b)) [56] and sorption characteristics (Figure 5.4a). Favourably, when meropenem was formulated as a ternary mix (i.e. SD-COL/MEM/TIG and SD-COL/MEM/RIF), the cohesiveness of meropenem was minimized. Capsule and device retention had decreased significantly to under 5% and 7%, respectively (Figure 5.5e and 5.5f). Clearly, it does seem that formulating the drugs into a ternary mix has the advantage of ameliorating the performance of the poorer-performing species. For the formulations with a crumpled sheet-like morphology (i.e. SD-RIF, SD-COL/MEM/RIF and SD-COL/TIG/RIF), the dispersibilities obtained were generally robust (i.e. FPFs ranging from $52.8 \pm 2.5\%$ to $69.5 \pm 5.0\%$), thus suggesting the favourability of the corrugated/crumpled particle morphology in aerodynamics [44, 57, 58]. These particles have high free volume with pockets of void space and hence have low densities, are aggregation-resistant, and have reduced inter-particle contact areas [44, 57, 58]. For the ternary spray-dried formulations (i.e. SD-COL/MEM/TIG, SD-COL/MEM/RIF and SD-COL/TIG/RIF), there was concomitant deposition observed across all stages (including the throat, inhaler and capsule), thus suggesting uniformity in the powder and/or aerosol (Figure 5.5e-g). Furthermore, all three ternary formulations are highly suited for delivery via the aerosol route in view of the robust FPFs obtained (Table 5.6.). Improvements to the emitted fraction (i.e. inferred via closely similar FPF and FPF (emitted)) were also achieved in the ternary species which again reinforces the benefits of co-delivery.

Figure 5.5. In vitro deposition of (a) SD-COL, (b) SD-MEM, (c) SD-TIG, (d) SD-RIF, (e) SD-COL/MEM/TIG, (f) SD-COL/MEM/RIF and (g) SD-COL/TIG/RIF at 60 L/min. Data presented as mean $\pm$ standard deviation (n=3). S1-S7 denote impactor stages, followed by their corresponding lower aerodynamic cutoff diameter in parentheses. MOC is the NGI micro-orifice collector. Reproduced with permission from Ref. [26]. Copyright 2016 Elsevier

Table 5.6. Deposition parameters of different formulations measured by NGI at 60 L/min^c

Formulations	FPF ^a (%)	FPF (emitted) ^b (%)
SD-COL	58.5 $\pm$ 2.1	69.4 $\pm$ 0.6
SD-MEM	42.6 $\pm$ 1.6	73.0 $\pm$ 1.4
SD-RIF	55.1 $\pm$ 3.7	63.6 $\pm$ 2.6
SD-TIG	56.0 $\pm$ 3.2	70.9 $\pm$ 2.2
SD-COL/MEM/TIG		
COL	61.2 $\pm$ 4.7	66.8 $\pm$ 6.2
MEM	64.9 $\pm$ 1.6	70.4 $\pm$ 2.2
TIG	62.2 $\pm$ 4.4	67.6 $\pm$ 5.2
SD-COL/MEM/RIF		
COL	55.9 $\pm$ 2.0	60.1 $\pm$ 2.3
MEM	52.8 $\pm$ 2.5	58.1 $\pm$ 2.1
RIF	54.2 $\pm$ 2.9	60.3 $\pm$ 2.8
SD-COL/TIG/RIF		
COL	69.5 $\pm$ 5.0	73.9 $\pm$ 6.1
TIG	67.2 $\pm$ 5.9	72.5 $\pm$ 6.4
RIF	67.8 $\pm$ 5.3	72.7 $\pm$ 5.9

^a FPF - fine particle fraction

^b FPF (emitted) - emitted fine particle fraction

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5.3.8. Antimicrobial activity of spray-dried powders

Pseudomonas aeruginosa is a leading opportunistic pathogen that is mostly responsible for hospital-acquired pneumonia (HAP) and ventilator-associated pneumonia (VAP) [59] that occurs predominantly in immunocompromised, burn and cystic fibrosis patients. This pathogen is particularly dangerous and dreaded due to its natural resistance to many antibiotics and its capacity to acquire new resistance mechanisms under pressure from antibiotics exposure [60]. In recent years, the incidence of resistance to the β -lactams, quinolones, aminoglycosides and carbapenems had risen steadily worldwide and at current growth rates, this phenomenon is undoubtedly poised to become a serious global medical threat [61-63].

Opportunistic *Pseudomonas aeruginosa* is implicated in many chronic infections and is readily isolated from delayed-healing wounds, medical devices, and the lungs of cystic fibrosis patients. Persistence of *Pseudomonas aeruginosa* is due to its capacity to form biofilms where bacteria embedded in biofilms is protected from antibiotic therapy [64, 65]. Antibiotic combinations had been shown to be more efficient than single antibiotics in treating biofilm-associated infections [66-68], and our colistin combinations are predicted to have the same enhanced anti-biofilm effects and hence improved antimicrobial activities against biofilm populations of *Pseudomonas aeruginosa*.

In this work, the antimicrobial activities of the ternary spray-dried powders (i.e. SD-COL/MEM/TIG, SD-COL/MEM/RIF and SD-COL/TIG/RIF) were tested against different MDR pathogenic respiratory bacteria and summarized in Table 5.7. Overall, the antibiotic combinations showed significant bactericidal effects, in particular, the SD-COL/TIG/RIF combination against *Pseudomonas*

aeruginosa, *Klebsiella pneumoniae* and *Escherichia coli*. Although the combinations' lack of synergistic effects on MDR *Acinetobacter baumannii* could be a strain-specific issue, they are nonetheless, as bactericidally-effective as colistin alone.

When all three ternary formulations were tested against MDR *Pseudomonas aeruginosa* that was resistant to ciprofloxacin (fluoroquinolone group), gentamicin (aminoglycoside group) and meropenem (carbapenem group), a synergistic kill was obtained across the formulations, with log₁₀ CFU changes of -4.0, -3.6 and -3.2 for SD-COL/TIG/RIF, SD-COL/MEM/TIG and SD-COL/MEM/RIF, respectively, after a 24 h test period. With a synergistic interaction, a much reduced drug dose would be required to achieve the same therapeutic effect as conventional monotherapy. This is in addition to both the combinatorial and local delivery strategies (directly to the lungs), which also served to reduce the required drug dose into the body. In totality, a synergistic inhaled combination would not only minimize the toxic side effects of the antibiotic/s (e.g. colistin), but also help in the minimization of drug resistance.

Although carbapenem-resistant bacteria is currently the bane of patients and medical practitioners worldwide [1, 2] and has limited treatment options, it is nevertheless very encouraging to note that meropenem (i.e. a carbapenem) is still relevant and potent in killing these resistant bacteria when applied in a combinatorial setting. Indeed, this could have positive implications in terms of extending the drug's useful 'lifespan'. Both SD-COL/MEM/RIF and SD-COL/MEM/TIG (carbapenem(MEM)-containing formulations) were not only bactericidally-effective against all four species of carbapenem-resistant bacteria

(Table 5.7), but also elucidated a synergistic response for the MDR *Pseudomonas aeruginosa* isolate.

Among the three ternary formulations developed in this work, SD-COL/TIG/RIF (non-carbapenem (MEM)-containing combination) was found to be the best performing species. It not only exhibited a synergistic interaction against carbapenem-resistant *Pseudomonas aeruginosa*, but also revealed additive interactions against other carbapenem-resistant Gram-negatives such as *Klebsiella pneumonia* and *Escherichia coli* (Table 5.7.). Although an additive interaction might be a notch lower than a synergistic interaction, they are nonetheless still useful in dosage reduction, which could help in the minimization of the associated side effects and the potential for bacterial resistance. The superior qualities of SD-COL/TIG/RIF (over SD-COL/MEM/RIF and SD-COL/MEM/TIG) may be attributed to colistin, tigecycline and rifampicin's distinct killing mechanisms which targeted different functions of the bacterial cell (Table 5.1. and Figure 5.1.). Bactericidal colistin is a cationic antibiotic that binds to the anionic lipopolysaccharide (LPS) of Gram-negative bacteria's membrane, leading to a disruption of the membrane permeability, leakage of intracellular content and hence cell death [69, 70]. Perturbation of the bacterial cellular membrane integrity by colistin may in turn favour the uptake of tigecycline and rifampicin to bring about a 'collaborative' synergistic interaction. Bactericidal rifampicin's mechanism of action is based on its ability to specifically bind and inhibit the bacterial DNA-dependent RNA polymerase [29]. On the other hand, bacteriostatic tigecycline works by binding to the 30S subunit of the bacterial ribosome, thus inhibiting peptide elongation and subsequently, disrupting protein synthesis [30]. As each of them (i.e. colistin, rifampicin and tigecycline) has a unique complementary

mode of action that is not shared by any other antibiotics in the combination, it is therefore not surprising to observe a potpourri of favourable interactions (i.e. synergy and additivity) arising from the SD-COL/TIG/RIF species (as compared to SD-COL/MEM/RIF and SD-COL/MEM/TIG) (Table 5.7.). For SD-COL/MEM/RIF and SD-COL/MEM/TIG, as they have two antibiotics (i.e. meropenem and colistin) with overlapping actions on the bacterial cell wall (Figure 5.1.), their ‘collaborative’ efficiency is therefore understandably, a notch lower than SD-COL/TIG/RIF.

Regular use of antibiotics, be it monotherapy or combination therapy, will eventually lead to resistance. The beauty of the present antibiotic arsenal lies in the potential for ‘rotational therapy’ (i.e. treatment rotated between the three different-member combinations). Recalcitrant bacterial infections that require prolonged antibiotic treatment could benefit from such a therapy while enjoying the reduced risk of antimicrobial resistance. With three unique combinations involving three different complementary antibiotics, the possibility of a bacterium developing resistance to all the antibiotics and combinations simultaneously would hence be very far remote. The present work has focused on, and successfully demonstrated the antimicrobial efficacy of the three ternary formulations on MDR bacteria. Future investigations will include in-vitro (mammalian cell lines) studies and in-vivo animal infection models. The results from these studies will help uncover potential cytotoxicity issues and provide pharmacokinetic insight into the drug combinations, helping to progress the product/s towards early phase human clinical trials.

Table 5.7. Time-kill results of spray-dried formulations against different MDR pathogenic respiratory bacteria^c

Resistant bacterial strain (Resistance profile)	Time-kill assay ^a (log ₁₀ change)	Interaction
<i>Pseudomonas aeruginosa</i> PSE2 (CIP ^R GEN ^R MEM ^R)		
SD-COL/TIG/RIF	-4.0	Synergy
SD-COL/MEM/TIG	-3.6	Synergy
SD-COL/MEM/RIF	-3.2	Synergy
<i>Klebsiella pneumoniae</i> ENT443 (CIP ^R MEM ^R) ^b		
SD-COL/TIG/RIF	-1.2	Additivity
SD-COL/MEM/TIG	0.1	Indifference
SD-COL/MEM/RIF	0.0	Indifference
<i>Escherichia coli</i> EC424 (CIP ^R MEM ^R) ^b		
SD-COL/TIG/RIF	-1.0	Additivity
SD-COL/MEM/TIG	0.1	Indifference
SD-COL/MEM/RIF	0.2	Indifference
<i>Acinetobacter baumannii</i> AB1 (CIP ^R GEN ^R MEM ^R)		
SD-COL/TIG/RIF	-0.5	Indifference
SD-COL/MEM/TIG	0.2	Indifference
SD-COL/MEM/RIF	0.2	Indifference

COL, colistin ; MEM, meropenem ; TIG, tigecycline; RIF, rifampicin; CIP, ciprofloxacin ; GEN, gentamicin ; MDR, multi-drug resistant

^a Values represent the log₁₀ change in CFU/ml in the time kill assay after 24-h exposure to the ternary spray-dried formulations compared to the most active drug

^b Isolate bears a carbapenemase which results in enzymatic hydrolysis of carbapenems conferring carbapenem resistance

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5.4. Conclusions

A ‘toolbox’ consisting of three potentially rotatable ternary DPI combinations (COL/TIG/RIF, COL/MEM/RIF and COL/MEM/TIG) was successfully tailor-made in the fight against respiratory ‘superbugs’. The spray-dried powder particles were of respirable-size (d_{50} of $3.1 \pm 0.3 \mu\text{m}$ to $3.4 \pm 0.1 \mu\text{m}$) and predominantly corrugated/crumpled in morphology. When dispersed via an Aerolizer[®] inhaler at 60 L/min, the powders showed concomitant *in vitro* deposition with obtained FPFs of ~53-70%. In this work, the benefits of co-delivery include the potential for synergy which helps to further lower the toxic drug dose and the associated side effects (i.e. colistin), ameliorating the

performance of a poorer-performing species (e.g. cohesiveness and dispersibility) (i.e. meropenem), the life-span extension of an ‘expiring/redundant’ antibiotic (i.e. meropenem) and the opportunity to mount a collaborative and concerted mode of attack on bacteria (i.e. COL/TIG/RIF). The advent of these novel formulations is indeed timely, and will no doubt equip the human race with the necessary tools to confront the ‘plague of the 21st century’ with renewed confidence.

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Chapter 6

Inhaled Non-Steroidal Natural Alternatives for Anti-Inflammatory Combination Therapy

6.1. Introduction

Airway inflammation is the central feature for respiratory disorders such as asthma and chronic obstructive pulmonary disease (COPD) [1, 2]. Despite asthma and COPD being pathologically distinct in terms of the inflammatory response characteristics and site of inflammation, both of them nevertheless still involve chronic inflammation of the respiratory tract, which is mediated by an increased expression of components of the inflammatory cascade including cytokines, chemokines, inflammatory enzymes and receptor [3].

Among the anti-inflammatory agents, inhaled corticosteroids are highly effective anti-inflammatory therapy in asthma, COPD and other chronic inflammatory and immune diseases [4, 5]. In fact, inhaled corticosteroids are always the first-line therapy in the control of persistent asthma in children and adult [6, 7]. Although the benefits of using inhaled corticosteroids in COPD is controversial [8], their utility in reducing COPD exacerbations and improving quality of life is nevertheless a highly promising and justified approach [9]. Unfortunately, there is a subset of patients who are found to be insensitive to corticosteroid therapy in their inflamed airway, even at high doses of inhaled and oral corticosteroid (i.e. corticosteroid -resistant) [4, 10]. Clinically, up to 50% of asthmatics have been reported to respond sub-optimally to corticosteroid therapy [11]. Furthermore, patients with COPD have also been frequently found to show a poor clinical response to corticosteroid therapy with a largely

corticosteroid-resistant pattern of inflammation [12]. Undoubtedly, the reduction in corticosteroid responsiveness (whether insensitivity or resistance) is a significant obstacle to the effective management and control of asthma and COPD.

Due to direct lung targeting effect, inhaled corticosteroid is typically used in much smaller doses than oral administration and generally regarded much safer for lengthy treatment. Nevertheless, the fear of inhaled corticosteroid (especially for prolonged use) to induce systemic adverse effects such as growth retardation in children and osteoporosis, skin thinning, cataracts, glaucoma and hypertension [13-17] has been a major concern for patients with airway inflammation. The systemic adverse effects with the use of inhaled corticosteroids both in children and adult have been well-documented [18, 19]. Besides systemic adverse effects, inhaled corticosteroids are also associated with local side effects such as perioral dermatitis, oral candidiasis infection, voice hoarseness, dysphonia, cough during inhalation, sore throat and a feeling of thirst [20, 21]. These local side effects are not only distressing but also affect patient compliance to the treatment.

Apparently, inhaled non-steroidal anti-inflammatory agents such as sodium cromoglycate and nedocromil offer possible alternatives for the management of airway inflammation with beneficial corticosteroid-sparing effects. However, they are often not regarded as effective alternatives to inhaled corticosteroids in view of their weak anti-inflammatory properties [22]. Although newer non-steroidal anti-inflammatory agents, including inflammatory mediators such as leukotriene modifiers are gaining popularity as a new class of asthma medication, they are unlikely to replace inhaled corticosteroids in the treatment

of asthma. As there are a large number of inflammatory mediators involved in asthma, blockade of one or a few mediators out of the over 50 different mediators that are identified in asthma is scarcely of clinical significance as compared to the non-specific agents (i.e. inhaled corticosteroids) [23, 24]. The efficacy and cost (more expensive than inhaled corticosteroids) challenges have hence hindered the widespread clinical use of leukotrienes as a first-line asthma therapy. Therefore, leukotriene modifiers are more commonly applied as adjunct therapy to the inhaled corticosteroids [25].

Owing to the limitations of clinically used anti-inflammatory drugs (both inhaled corticosteroids and inhaled non-steroidal anti-inflammatory agents), natural agents and traditional herbal medicines appear to be a highly attractive alternative to patients showing chronic inflammation of the airways. They have the benefits of a lower cost, widespread availability and better side effect profile. In fact, plants products (whether as parts of foods or botanical potions and powders) have been a source of therapeutic agents throughout the world for over 5000 years [26]. To date, over 85,000 plant species worldwide have been documented useful medicinally [27]. According to the World Health Organization (WHO), approximately 75% of the world's population has some sort of therapeutic experience with herbal remedies [28] and as much as 25% of modern pharmaceutical drugs contain one or more ingredients derived from plants [29]. In light of these statistics, natural agents are expected to continue providing mankind with more new and novel remedies. In fact, a large number of plants and herbs with anti-inflammatory activity have long been folk remedies against various inflammatory disorders which include, but are not limited to asthma [30].

Polyphenols, the biggest class of phytochemical [31] that can be found abundantly in most dietary sources such as fruits and vegetables, have been shown to exert potent anti-inflammatory activity [30, 32]. Curcumin, quercetin, resveratrol and many others, are among the polyphenols that have been shown to modulate pro-inflammatory gene expressions [33-35]. In the recent years, there has been strong interests in exploiting their therapeutic qualities for the treatment of airway inflammations. These polyphenols are also reputed for their anti-oxidant [36-38] and anti-fungal [39-41] properties to counteract the fungal infections and oxidative stress that are very likely to develop in the inflamed lung. Therefore, these compounds have the potential to be applied as either mainstream or adjunct therapies for airway inflammation. Curcumin (chemical structure as shown in Figure 5.1a) is a yellow pigment derived from the root of the *Curcuma longa* plant which has been consumed by people for centuries as a dietary component. Impact of curcumin in the treatment of chronic inflammation in asthmatics and patients with COPD has been demonstrated through recent research observations that curcumin is able to decrease TNF- α -induced inflammation [42, 43], block the activation of extracellular signal-regulated kinase pathway [44], restore the efficacy of glucocorticoids by inducing HDAC2 activity [45], inhibit activation of NF- κ B [33, 46] and the Notch-GATA3 signaling pathway [47]. Quercetin (chemical structure as shown in Figure 5.1b) is a polyphenolic flavonoid that is found ubiquitously in many brightly-colored fruits and vegetables such as apples, tomatoes, onions and teas. Quercetin has been renowned to act as a natural anti-histamine to inhibit the release of histamine, an important mediator in airway inflammation, from mast cells [48, 49]. Several epidemiological studies had demonstrated the potential of quercetin to modulate the early and late phases of asthma, by suppressing the

activation of eosinophil, inhibiting GATA-3 and increasing T-bet expression [49-51]. Resveratrol (chemical structure as shown in Figure 5.1c) is an important natural occurring stilbene class of the polyphenols which found prevalently in grapes, red wines and some berries. Resveratrol exists in two isomeric forms: trans-resveratrol and cis-resveratrol, with trans form being more stable and potent [44]. It has been reported that resveratrol exhibits its anti-inflammatory properties through inhibition of the cyclooxygenase (COX)-2 transcription and COX-1 activity via peroxidase-mediate mechanism [52], inhibition of transcription factors such as NF- κ B and activator protein-1 (AP-1), which is also a potent regulator of oxidative stress related genes [53] and activation of the peroxisome proliferator-activated receptor (PPAR)- α [54]. Several in vivo evidences of anti-inflammatory action of curcumin, quercetin and resveratrol in a chronic allergic airway disease model had also been reported in the literature [44, 47, 55-57].

Figure 6.1. Chemical structures of (a) curcumin, (b) quercetin and (c) resveratrol

Although there had been a number of studies investigating the anti-inflammatory effect of curcumin, resveratrol and quercetin in airway diseases, most of them were only reported as oral therapy. Administration of these natural agents through inhalation had received little consideration and only occasionally been reported [58-63]. A combination of natural agents with different mechanisms of action at much lower and safer dosages appears to be a potentially attractive treatment option. To the best of the authors' knowledge, no combined nutraceutical-based dry powder inhaler formulation has been reported in the academic literature.

6.2. Aim

The aim of this work was to pioneer a novel inhalable non-steroidal nutraceutical-based dry powder formulation incorporating curcumin, quercetin and trans-resveratrol (namely, SD-CUR/QUER/RES) for anti-inflammatory combination therapy via state-of-the-art nanospray drying. Dry powder inhalers (DPIs) are gaining momentum in the local delivery of therapeutic agents to the lung, as they are more portable with faster delivery and have better delivery efficiency than nebulisers, and powdered drugs are more stable than the medicine solution or suspension in the metered dose inhaler (MDI) and nebulizer which typically require cold chain storage [64-66]. In the present study, amino acid L-leucine (LEU) was used as a hydrophilic stabilizer to enhance the dispersibility and dissolution rate of the formulation. The physiochemical properties, in vitro aerosolization performance and in vitro dissolution behaviour and effect of the ternary formulations comprising

curcumin, quercetin and trans-resveratrol (with and without L-leucine) on inflammatory mediator release were evaluated.

6.3. Results and discussion

6.3.1. Particle size, surface morphology and internal structure

The surface morphologies of raw curcumin (Raw-CUR), quercetin (Raw-QUER) and trans-resveratrol (Raw-RES) and ternary spray-dried particles (i.e. SD-CUR/QUER/RES, SD-CUR/QUER/RES/LEU-25 and SD-CUR/QUER/RES/LEU-50) are shown in Figure 6.2. Raw-CUR (Figure 6.2a) and Raw-RES (Figure 6.2c) appeared as irregularly shaped chips and chunks while Raw-QUER was in irregular rod-like shape (Figure 6.2b). In contrast, the ternary spray-dried powders consisted of particles that have more uniform shapes and sizes. They were generally spherical in geometry with different degree of roundness and surface roughness. Basically, SD-CUR/QUER/RES particles changed from slightly rough spherical (Figure 6.2d) to a smooth spherical (Figure 6.2e) and then to a wrinkly raisin-like and donut-shaped (Figure 6.2f) morphology with the addition of increasing amounts of L-leucine.

Figure 6.2. FESEM images of raw materials and spray-dried powders. (a) Raw-CUR, (b) Raw-QUER, (c) Raw-RES, (d) SD-CUR/QUER/RES, (e) SD-CUR/QUER/RES/LEU-25 and (f) SD-CUR/QUER/RES/LEU-50

The internal morphologies of the ternary spray-dried particles were examined by FIB/SEM. The cross sections of particles were first produced by FIB milling

and then the internal structures of the particles were observed under SEM. Figure 6.3. shows SEM images of FIB-milled cross-sections of SD-CUR/QUER/RES, SD-CUR/QUER/RES/LEU-25 and SD-CUR/QUER/RES/LEU-50 particles. Both SD-CUR/QUER/RES and SD-CUR/QUER/RES/LEU-25 particles showed solid interior (Figure 6.3a & 6.3b) whereas SD-CUR/QUER/RES/LEU-50 particles revealed hollow interior (Figure 6.3c). The difference in surface morphology and internal structure of SD-CUR/QUER/RES/LEU-50 particles could be explained by the surface activity of L-leucine. As surface active agents, L-leucine molecules tend to accumulate and precipitate instantaneously at the air-water interface, forming a thin layer on the surface of water droplets and leading to shell or skin formation. Due to the rapid formation of the L-leucine surface layer, the droplets further expand like a balloon, leaving a hollow void towards centre as the solvent evaporation progresses. Upon complete solvent evaporation, the hollow particle may eventually collapse into a wrinkled/donut structure [67]. No wrinkled (i.e. punctured), donut-shaped (i.e. collapsed) or hollow particles were observed for SD-CUR/QUER/RES/LEU-25 particles. This could possibly be due to the amount of L-leucine in the formulation not being at the required threshold levels to stimulate shell formation and the eventual punctured or collapsed structure.

Figure 6.3. Secondary electron images of the FIB cross-sections of (a) SD-CUR/QUER/RES, (b) SD-CUR/QUER/RES/LEU-25 and (c) SD-CUR/QUER/RES/LEU-50 after FIB milling

Table 6.1. shows the particle size distribution of the ternary spray-dried powders. The particle size analysis revealed that all the spray-dried formulations were within the respirable size range (i.e. d_{50} of 2.12 ± 0.22 , 2.07 ± 0.09 and 1.95 ± 0.03 , for SD-CUR/QUER/RES, SD-CUR/QUER/RES/LEU-25 and SD-CUR/QUER/RES/LEU-50, respectively) with a narrow size distribution (span < 2) (Table 6.1.).

Table 6.1. Volume particle size distribution (mean $\pm$ SD, n=3) of the spray-dried powders

Formulations	d ₁₀ (μm)	d ₅₀ (μm)	d ₉₀ (μm)	Span
SD-CUR/QUER/RES	1.08 $\pm$ 0.09	2.12 $\pm$ 0.22	4.26 $\pm$ 0.69	1.46 $\pm$ 0.13
SD-CUR/QUER/RES/LEU-25	1.06 $\pm$ 0.09	2.07 $\pm$ 0.09	4.24 $\pm$ 0.81	1.53 $\pm$ 0.39
SD-CUR/QUER/RES/LEU-50	0.96 $\pm$ 0.02	1.95 $\pm$ 0.03	4.30 $\pm$ 0.39	1.72 $\pm$ 0.19

d₁₀ - volume diameter under which 10% of the sample residesd₅₀ - volume median diameterd₉₀ - volume diameter under which 90% of the sample residesSpan = (d₉₀ - d₁₀) / d₅₀

6.3.2. Powder crystallinity

The diffractogram of the raw materials and ternary spray-dried powder is shown in Figure 6.4. Unprocessed materials which included raw curcumin (Raw-CUR), raw quercetin (Raw-QUER), raw trans-resveratrol (Raw-RES) and raw L-leucine (Raw-LEU) individually showed multiple sharp peaks characteristic of crystalline materials. The sharp XRD Bragg peaks of L-leucine had diminished in intensity after spray-drying, indicating the reduced crystallinity of SD-LEU as compared to Raw-LEU. The SD-CUR/QUER/RES spray-dried powder did not exhibit any of the characteristic reflections of the starting materials (i.e. Raw-CUR, Raw-QUER and Raw-RES), instead displayed characteristic amorphous diffused halo. In the co-spray dried powders with presence of L-leucine (i.e. SD-CUR/QUER/RES/LEU-25 and SD-CUR/QUER/RES/LEU-50), crystalline peaks of L-leucine at $\sim 6^\circ$ and $\sim 19^\circ$ (as observed in SD-LEU) appeared and increased in intensity with the increased of L-leucine content, in addition to the diffused halo (Figure 6.4b).

Figure 6.4. Zoom-in XRD patterns of (a) Raw-CUR, Raw-QUER, Raw-RES, SD-LEU, SD-CUR/QUER/RES, SD-CUR/QUER/RES/LEU-25 and SD-CUR/QUER/RES/LEU-50 and (b) SD-LEU, SD-CUR/QUER/RES, SD-CUR/QUER/RES/LEU-25 and SD-CUR/QUER/RES/LEU-50

6.3.3. Specific surface area

The specific surface areas for the starting materials and ternary spray-dried formulations are shown in Table 6.2. The high specific surface area of Raw-QUER (i.e. $6.67 \pm 0.54 \text{ m}^2/\text{g}$) compared to other starting materials may be due to its rod-like shape of morphology. Typically, particle with elongated shape has a larger surface-area-to-volume ratio than its spherical counterpart [68]. Among the ternary spray-dried formulations, SD-CUR/QUER/RES/LEU-50 has the highest specific area (i.e. $7.94 \pm 0.16 \text{ m}^2/\text{g}$) in comparison to ternary formulation with lower L-leucine content (i.e. SD-CUR/QUER/RES/LEU-25) and without L-leucine content (SD-CUR/QUER/RES). The higher specific area of SD-CUR/QUER/RES/LEU-50 may be attributed to the external and internal features of the particles (i.e. wrinkled, donut-shaped, hollowed particles) that were determined by SEM and FIB-SEM.

Table 6.2. Specific surface areas of the spray-dried formulations

Formulations	Specific surface area (m^2/g)
Raw-LEU	1.25 ± 0.24
Raw-CUR	2.03 ± 0.24
Raw-QUER	6.67 ± 0.54
Raw-RES	2.60 ± 0.37
SD-CUR/QUER/RES	2.88 ± 0.13
SD-CUR/QUER/RES/LEU-25	4.00 ± 0.58
SD-CUR/QUER/RES/LEU-50	7.94 ± 0.16

6.3.4. Moisture sorption profiles

The effect of the humidity on moisture sorption of ternary spray-dried formulations was investigated. Figure 6.5 shows the moisture sorption isotherms of the raw materials (Figure 6.5a) and ternary spray-dried

formulations (i.e. SD-CUR/QUER/RES, SD-CUR/QUER/RES/LEU-25 and SD-CUR/QUER/RES/LEU-50) (Figure 6.5b). Among all the raw materials, Raw-QUER exhibited the highest maximum mass change of approximately 9%, at 90% RH (Figure 6.5a). The desorption isotherm of Raw-QUER from 90% RH to 0% RH showed an irreversible hysteresis, indicating the hygroscopicity of the material. As Raw-QUER showed partial crystallinity only (Figure 6.4a), it is most likely that this material swelled slowly and irreversibly during water sorption and retained some absorbed moisture on desorption completion. In contrast, both Raw-LEU and Raw-CUR exhibited reversible water sorption profiles with low adsorption of water even at elevated humidity levels. For the ternary spray-dried powder, both SD-CUR/QUER/RES and SD-CUR/QUER/RES/LEU-25 exhibited higher moisture sorption profile over the range of 0 to 60% RH as compared to SD-CUR/QUER/RES/LEU-50. Furthermore, both SD-CUR/QUER/RES and SD-CUR/QUER/RES/LEU-25 showed a mass loss after RH reached approximately 60% and 70%, respectively. These losses of mass corresponded to the expulsion of absorbed water from the samples during recrystallization as a consequence of the plasticizing effect of water on the amorphous materials [69]. In contrast, SD-CUR/QUER/RES/LEU-50 showed no evidence of recrystallization. This was probably due to the moisture protective effects of L-leucine [70] when administered at an optimal amount.

Figure 6.5. DVS isotherm of the (a) raw materials and (b) ternary spray-dried formulations

6.3.5. In vitro aerosol performance

The aerosolization performance results of the ternary spray-dried formulations are shown in Figure 6.6. and Table 6.3. In this work, airflow of 60 L/min was used to simulate the reduced inspiratory effort achieved by patients with compromised lung functions (e.g. asthma and COPD) [71, 72]. The FPFs for

SD-CUR/QUER/RES was ~12% ($12.98 \pm 1.2\%$ for curcumin, $12.19 \pm 1.34\%$ for quercetin and $11.52 \pm 1.56\%$ for trans-resveratrol), with a significant powder deposition on stage 1 ($32.67 \pm 5.33\%$ for curcumin, $33.18 \pm 5.54\%$ for quercetin and $34.21 \pm 6.04\%$ for trans-resveratrol) and throat ($24.08 \pm 3.20\%$ for curcumin, $24.71 \pm 3.32\%$ for quercetin and $25.56 \pm 4.31\%$ for trans-resveratrol). The high powder deposition on stage 1 and throat is presumably due to the agglomeration of the powder. With the addition of 25 wt.% of L-leucine to the formulation (i.e. SD-CUR/QUER/RES/LEU-25), the deposition on stage 1 could be reduced to ~22% ($22.86 \pm 1.60\%$ for curcumin, $23.64 \pm 1.77\%$ for quercetin and $21.66 \pm 2.56\%$ for trans-resveratrol). When the L-leucine content in the formulation has further increased to 50 wt.%, the powder deposition on stage 1 has been significantly reduced to ~4% ($4.30 \pm 0.36\%$ for curcumin, $4.59 \pm 0.46\%$ for quercetin and $3.47 \pm 0.34\%$ for trans-resveratrol). The ternary formulation SD-CUR/QUER/RES/LEU-50 showed up to 3.5-3.8 enhancement in total FPF over SD-CUR/QUER/RES. The poor FPF of SD-CUR/QUER/RES may be due to the strong interparticulate cohesion (i.e. van der Waals and electrostatic forces) within the micron dimension of the spherical drug particles which inhibit particle detachment especially when they exit the inhaler. Whereas, the surface asperities of L-leucine coated SD-CUR/QUER/RES/LEU-50 reduced powder cohesiveness by minimizing the contact area between adjacent particle surfaces.

Figure 6.6. In vitro deposition of spray-dried powders at 60 L/min. (a) SD-CUR/QUER/RES, (b) SD-CUR/QUER/RES/LEU-25 and (c) SD-CUR/QUER/RES/LEU-50. S1-S4 denote impactor stages, followed by their corresponding aerodynamic cut-off diameter in parentheses

Table 6.3. Deposition parameters (mean $\pm$ standard deviation, n = 3) of different formulations measured by MSLI at 60 L/min

	<u>SD-CUR/QUER/RES</u>			<u>SD-CUR/QUER/RES/LEU-25</u>			<u>SD-CUR/QUER/RES/LEU-50</u>		
	CUR	QUER	RES	CUR	QUER	RES	CUR	QUER	RES
FPF ^a (%)	12.98 $\pm$ 1.20	12.19 $\pm$ 1.39	11.52 $\pm$ 1.56	26.95 $\pm$ 2.38	25.76 $\pm$ 2.06	24.99 $\pm$ 4.40	45.70 $\pm$ 1.33	43.31 $\pm$ 1.00	43.91 $\pm$ 2.31
FPF (emitted) ^b (%)	16.45 $\pm$ 1.48	15.51 $\pm$ 1.68	14.63 $\pm$ 1.80	32.63 $\pm$ 2.26	31.10 $\pm$ 1.90	30.28 $\pm$ 4.66	57.12 $\pm$ 1.92	54.55 $\pm$ 1.57	56.27 $\pm$ 3.45

^a FPF - fine particle fraction

^b FPF (emitted) - emitted fine particle fraction

6.3.6. Powder dissolution

Despite their well-known anti-inflammatory properties, quercetin, curcumin and resveratrol can be poorly soluble in water, with reported aqueous solubility of 0.3 µg/ml [73], 0.4 µg/ml [74] and 21 µg/ml [73] at 37°C, respectively. The low aqueous solubility could limit the dissolution rates and lead to a slow absorption. Compounds with such low solubilities typically exhibit dissolution rate-limited absorption [75]. Hence, improvements to the compound's pulmonary dissolution rates would be beneficial to the absorption process in the lungs. Figure 6.7 shows the dissolution profiles of ternary formulation consisting of curcumin, quercetin and trans-resveratrol, with (i.e. SD-CUR/QUER/RES/LEU-25 and SD-CUR/QUER/RES/LEU-50) and without L-leucine (i.e. SD-CUR/QUER/RES). With the incorporation of L-leucine into the ternary formulation, the dissolutions rate and extent of quercetin, curcumin and trans-resveratrol was found to be much improved over their counterparts in the formulation without L-leucine. For quercetin, its extent of dissolution (at 300 min) was increased by approximately 3.7 times from $3.5 \pm 0.5\%$ (for quercetin in SD-CUR/QUER/RES) to $12.9 \pm 4.3\%$ (for quercetin in SD-CUR/QUER/RES/LEU-50) (Figure 6.6d). On the other hand, curcumin has a 2.2-fold increase in dissolution extent (at 300 min) from $13.3 \pm 1.3\%$ (for curcumin in SD-CUR/QUER/RES) to $31.5 \pm 2.1\%$ (for curcumin in SD-CUR/QUER/RES-50L) (Figure 6.7e). Trans-resveratrol also demonstrated a 1.5-fold increase in its dissolution extent (at 300 min) from $49.0 \pm 4.3\%$ (for resveratrol in SD-CUR/QUER/RES) to $73.6 \pm 3.1\%$ (for resveratrol in SD-CUR/QUER/RES/LEU-50) (Figure 6.7f). From the dissolution data obtained, quercetin was consistently found to be the as least aqueous soluble compound in the ternary formulation follow by curcumin and trans-resveratrol (Figure

6.7a-c). This finding correlates well with the literature solubility data as aforementioned [73, 74]. Incorporation of water-soluble L-leucine to the ternary formulation appeared to be highly effective in “lifting” the performance of the poorly performing species (i.e. quercetin, curcumin and trans-resveratrol). The release mechanism in this case was analogous to an “excipient-API solid dispersion system” whereby the poorly water-soluble components was dispersed into a more water-soluble L-leucine matrix, hence enhancing the wettability and solubility of the poorly water-soluble components. We had previously reported a similar approach to enhance the dissolution rate of a poorly water component in a binary formulation [72].

Figure 6.7. In vitro dissolution rate profiles of (a) SD-CUR/QUER/RES, (b) SD-CUR/QUER/RES/LEU-25, (c) CUR/QUER/RES/LEU-50, (d) quercetin in various ternary combination formulations, (e) curcumin in various ternary combination formulations and (f) trans-resveratrol in various ternary combination formulations

6.3.7. Effect of ternary formulations and related compounds on inflammatory mediator release from A549 cells

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a 15 to 40 kD acid glycoprotein that promotes the differentiation and maturation of granulocytes and macrophages from progenitor cells [76]. GM-CSF is part of the natural immune and inflammatory cascade that serves to perpetuate inflammation reactions. In normal airways, GM-CSF is expressed at low or undetectable level but elevated levels of GM-CSF are often detected abundantly in allergic airways [77]. GM-CSF has profound effects on eosinophils, which are one of the most proinflammatory cells associated with the pathogenesis of allergic airway inflammation, by accelerating the growth and maturation of eosinophils as well as priming them for activation and enhancing their survival rates [78, 79].

We investigated the effect of the optimum ternary formulations (i.e. SD-CUR/QUER/RES/LEU-50) on GM-CSF release from A549 cells in comparison to the classical corticosteroids (i.e. budesonide (BD) and beclomethasone dipropionate (BP)), as well as a herbal anti-inflammatory preparation, Prospan[®]. Prospan[®] is a registered German herbal drug based on ivy leaf extract for treating chronic inflammatory bronchial diseases such as bronchitis, asthma, bronchiectasis, emphysema, pneumonia and acute inflammation of the respiratory tract accompanied by coughing [80]. The reported benefits of ivy leaf extract range from promoting healing and reducing inflammation [81], to treating asthma and COPD [82-84]. In this study, A549 (human lung adenocarcinoma epithelial) cell line was used as it has been widely chosen as an in vitro cellular model system for studying bronchial epithelial inflammation in

asthma due to the behavioral similarity between A549 and normal epithelial cell [85].

Figure 6.8. shows that both budesonide (~50 µg/ml) and beclomethasone dipropionate (~50 µg/ml) (as positive controls) inhibited GM-CSF release from A549 cells by approximately 35%, whereas Prospan[®] (at three different concentration levels: ~16.25 µg/ml (¼ tablet), ~32.5 µg/ml (½ tablet) and ~65.0 µg/ml (1 tablet), within range of the equivalent concentration of BP and BD) had no significant inhibitory effect.

Favourably, SD-CUR/QUER/RES/LEU-50 (CUR/QUER/RES concentration ≈ 50 µg/ml) exhibited higher inhibition of GM-CSF release from A549 cells over that of a commercial comparable herbal preparation, Prospan[®] and a similar inhibition of GM-CSF release from A549 cells (~30% of inhibition) over the mainstream corticosteroid (i.e. budesonide and beclomethasone dipropionate). A herbal preparation (i.e. Prospan[®]) when used at ~16.25 µg/ml (¼ tablet) had no inhibitory effect on GM-CSF release from A549 cells. There was still no inhibitory effect on GM-CSF release from A549 cells when the concentration of Prospan[®] had increased to ~65.0 µg/ml (1 tablet). In contrast, SD-CUR/QUER/RES/LEU-50 (with concentration of CUR/QUER/RES) at ~ 50 µg/ml had been demonstrated to affect GM-CSF release from A549 cells. These results suggested that the combinations of curcumin, quercetin and resveratrol into a co-spray dried formulation could be as potent as the mainstream corticosteroids in inhibiting GM-CSF release from cells for airway inflammation treatment. Therefore, our optimum ternary formulation, SD-CUR/QUER/RES/LEU-50, may potentially be used as sole therapy in airway inflammation or as complementary corticosteroid-sparing therapy facilitating

patients with inflamed airway to take smaller or shorter courses of corticosteroids.

Figure 6.8. Effect of SD-CUR/QUER/RES/LEU-50 on the release of GM-CSF from A549 as compared to mainstream corticosteroid (budesonide and beclomethasone dipropionate) and herbal anti-inflammatory preparation (Prospan[®])

6.4. Conclusion

The current work presents a dry powder inhalable formulation consisting of three nutraceuticals with multiple therapeutic properties. The optimum inhalable ternary formulation (SD-CUR/QUER/RES/LEU-50), containing curcumin, quercetin and trans-resveratrol, was successfully produced by co-spray drying with a suitable L-leucine content. The incorporation of 50 wt.% L-leucine into the ternary formulation (1) markedly improved the aerosolization performance of the spray-dried powders; (2) shielded the amorphous actives from recrystallization up to RH 90% at 25°C; (3) enhanced the dissolution rate of the poorly water-soluble curcumin, quercetin and trans-resveratrol actives. In

the vitro studies, SD-CUR/QUER/RES/LEU-50 exhibited anti-inflammatory activity that is on par with mainstream corticosteroid and superior to comparable herbal anti-inflammatory preparation (e.g. Prospan[®]) in inhibiting the release of GM-CSF from A549 cells. Therefore, a co-formulated powder of curcumin, quercetin and trans-resveratrol could be used as a potential non-steroidal nutraceutical alternative for the treatment of chronic inflammatory lung diseases such as asthma and COPD.

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Chapter 7

Conclusion and Recommendation

7.1. Summary

Combination therapies, which are important innovations in pharmacotherapy [1], are increasingly applied in treating a variety of local and systemic diseases. With the clinical-proven benefits of combination therapies (e.g. ICS/LABA, SABA/SAMA, LABA/LAMA and ICS/LABA/LAMA combinations) in the management of asthma and COPD, it is anticipated that there will be a trend towards the use of combination therapy in inhaled drug delivery. Among the inhaled combination therapies, inhaled combination antimicrobial therapy is becoming increasingly important, particularly in the drought of newer antibiotic discoveries. Binary and ternary DPI antibiotic formulations with/without non-antibiotic adjuvants have been developed and studied in this thesis for the treatment of various respiratory system infections. In view of the belief that too much mainstream medication is not always good for human health, nutraceuticals have therefore in recent years received considerable interest as an attractive alternative to these mainstream pharmaceuticals. There is a growing trend in nutraceutical usage for improving health and preventing chronic disorders. Therefore, exploitation of combination natural agents in aerosolized form is worthy for further attention as a “safer” and “cheaper” treatment alternative to deal with airway inflammation and as an attractive alternative therapy in dealing with mainstream steroidal drug resistance. The major conclusions in this work are summarized as follows.

Chapter 3

1. A steroid-antibiotic combination offers an innovative and realistic therapeutic option for CAP patients with underlying medical conditions such as COPD.
2. A novel dry powder inhaler formulation of an antibiotic (ciprofloxacin hydrochloride, CIP) and a corticosteroid (beclomethasone dipropionate, BP) for direct concomitant delivery to the lung was successfully produced via spray drying.
3. The resulting combination powder (SD-BP/CIP) was in the inhalable size range and displayed appropriate morphology for inhalational drug delivery.
4. Both BP and CIP in the resulting combination powder exhibited better drug release profile and aerosol properties (i.e. higher FPFs) than the powders of antibiotic or corticosteroid spray-dried alone.
5. SD-BP/CIP also showed robust in vitro activity against the respiratory tract infection-causing bacteria *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*.
6. The preliminary results demonstrated that SD-BP/CIP is a novel anti-inflammatory and antimicrobial formulation, which could potentially be used for treating CAP patients complicated with COPD.

Chapter 4

1. The accumulation of thickened mucus secretions in the lower airways provides a nutrient-rich breeding ground for bacteria that promotes their growth and limits the ease of effective eradication.

2. A novel ternary antibiotic-mucoactive agent dry powder inhaler formulation that comprises of ambroxol hydrochloride (AMB, a multi-faceted molecule with properties such as quorum quenching [2-4] and mucoactivity [5]) and the synergistic-antibiotic pair (CIP, ciprofloxacin hydrochloride and GAT, gatifloxacin hydrochloride) was successfully produced via spray-drying to facilitate simultaneous treatment and clearance of respiratory bacteria and mucus, respectively.
3. The resulting combination powder of CIP, GAT and AMB (SD-CIP/GAT/AMB/LEU) was in the inhalable size range and displayed appropriate morphology for inhalational drug delivery.
4. All CIP, GAT and AMB in the resulting combination powder showed much higher and uniform FPFs than the powders of antibiotics or mucoactive agent spray-dried alone.
5. The faster bacterial killing rate of the optimized ternary formulation (SD-CIP/GAT/AMB/LEU) in bacterial-spiked ASM than just the synergistic antibiotics alone (SD-CIP/GAT) demonstrated the influence of mucoactive agent in improving drug penetration and destruction of the biofilm-caged bacteria.
6. The preliminary results demonstrated that SD-CIP/GAT/AMB/LEU is a ternary antibiotic-(non-antibiotic) DPI formulation that is able to deliver a unique multi-pronged attack (synergy, quorum quenching and mucociliary clearance) on mucus-coated bacteria. This could prove useful in the treatment of mucoid infections.

Chapter 5

1. Respiratory lung infections due to multidrug-resistant (MDR) superbugs are on a global upsurge and their multidrug-resistant profile makes therapeutic options extremely limited.
2. Three potentially rotatable ternary DPI constructs (each involving colistin and two other different-classed antibiotics chosen from rifampicin, meropenem and tigecycline) were identified (with distinct complementary killing mechanisms) and co-formulated via spray-drying in the fight against respiratory “superbugs”.
3. The powder particles were of respirable-size and predominantly crumpled in morphology with good aerosol performance.
4. A favourable synergistic interaction was detected across all three formulations when tested against MDR-*Pseudomonas aeruginosa*.
5. These three ternary DPI combinations (COL/TIG/RIF, COL/MEM/RIF and COL/MEM/TIG) have the potential to offer improved efficacies at much reduced colistin doses and related adverse effects.

Chapter 6

1. Although inhaled corticosteroids have proven clinically to be effective and safer than oral corticosteroids, the prolonged use of high doses of inhaled corticosteroids increases the risk of inducing corticosteroid resistance and systemic adverse effects.
2. A novel inhalable ternary formulation, containing natural agents curcumin, quercetin and trans-resveratrol, was successfully produced by co-spray drying with a suitable L-leucine content (i.e. 50 wt.%) as a

nutraceutical-based combination powder for inhaled anti-inflammatory therapy.

3. The resulting combination powder (SD-CUR/QUER/RES/LEU-50) was in inhalable size range, had a wrinkled surface and porous structure that was indicative of a low relative density, making their aerodynamic properties highly favorable.
4. SD-CUR/QUER/RES/LEU-50 exhibited a high aerosolization performance, enhanced dissolution rate of poorly water-soluble curcumin, quercetin and trans-resveratrol, improved physical stability against moisture-induced recrystallization and anti-inflammatory activity in inhibiting the release of GM-CSF from A549 cells.
5. The data presented, while preliminary, provide evidence that delivery of natural agents (i.e. curcumin, quercetin and trans-resveratrol) in combination via DPI is feasible and could potentially be used as a cheap and safe non-steroidal alternative to treat inflammatory airways disorders (e.g. asthma and COPD).

7.2. Future work

Several novel combination dry powder formulations have been developed and studied in this thesis for the treatment of respiratory system infections and airway inflammation. Most of the combination dry powder formulations developed have demonstrated good physicochemical properties, in vitro aerosol performance and in vitro efficacy. While the findings in this thesis show promise, more work is still required to gain further insights into each formulation.

As the stability of the formulation dictates the shelf-life of the marketed product and determines its recommended storage conditions, it is therefore vital to evaluate the long term (real-time) and accelerated stability of each novel formulation. Powders representing the three batches could be filled into the capsules and then placed in the Activ-Vials[®] (to simulate the actual blister packaging) for long term (25°C and 60% RH for 12 months) and accelerated (40°C and 75% RH for 6 months) stability studies, based on the guidelines from International Conference of Harmonization [6] . In accelerated stability testing, the formulation is to be stored at elevated stress conditions (at relatively high temperatures and/or humidity) for enhanced mechanistic understanding of physicochemical stability and shelf-life of drug products [7]. At each time point (e.g. 0, 3, 6, 12, 18 and 24 months for long term stability studies and 0, 1, 3 and 6 months for accelerated stability studies), powders are to be evaluated for the effect of storage conditions on particle morphology, particle size, powder crystallinity and in vitro aerosol performance.

The present in-vitro work has demonstrated the efficacy of formulations in the treatment of respiratory system infection and airway inflammation. However, it is imperative to extend the research into the in-vivo system, in particular, the human body to understand the basis of mechanism of action of drug particle at both the cellular and molecular levels. In-vitro systems are generally regarded as “static” as compared to a much more complicated in-vivo dynamic system. Hence, discrepancies between in-vitro and in-vivo drug test results are not uncommon. The in vivo efficacy of the novel formulations could first be investigated through the rabbit model of bacterial lung infection and airway inflammation.

Both rabbits and sheep could be good animal models to study our novel combination dry powder formulations as the airway anatomy and responses to inflammatory mediators of rabbit are very similar to human [8] and the size and generations of sheep lungs reasonably correlate with human lungs [9]. The combination powders of this thesis could be administered via an insufflations device (PennCentury, Philadelphia, PA) into the rabbit or sheep lungs. Following dry powder insufflation, the pharmacokinetics profiles of inhaled powders could be determined in blood, lung tissue and bronchoalveolar lavage (BAL). Blood, BAL fluid and lung tissue could also be sampled to determine microbiological counts or to quantify inflammatory mediators.

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Appendix A

Published Journal Papers

Steroid-Decorated Antibiotic Microparticles for Inhaled Anti-Infective Therapy

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ABSTRACT: Despite advances in vaccination and antimicrobial therapy, community-acquired pneumonia (CAP) remains as a leading cause of morbidity and mortality worldwide. As the severity of CAP has been linked to the extent of inflammation in the body, adjunctive therapeutic measures aimed at modulating the immune response have therefore become increasingly attractive in recent years. In particular, for CAP patients with underlying medical conditions such as chronic obstructive pulmonary disease (COPD), a steroid–antibiotic combination will no doubt be a useful and timely therapeutic intervention. Unfortunately, no combined steroid–antibiotic dry powder formulation is available commercially or has been reported in the academic literature. The aim of this work was hence to develop a novel steroid–antibiotic dry powder inhaler formulation [ciprofloxacin hydrochloride (CIP) and beclomethasone dipropionate (BP)] for inhaled anti-infective therapy. The spray-dried powder was of respirable size (d_{50} of $\sim 2.3 \mu\text{m}$), partially crystalline and had BP preferentially deposited on the particle surface. Favorably, when formulated as a binary mix, both CIP and BP showed much higher drug release and fine particle fractions (of the loaded dose) over their singly delivered counterparts, and had robust activity against the respiratory tract infection-causing bacteria *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association *J Pharm Sci* 103:1115–1125, 2014

Keywords: dry powder inhaler; community-acquired pneumonia (CAP); chronic obstructive pulmonary disease (COPD); corticosteroids; combinatorial therapy; spray drying; aerosols; pulmonary drug delivery; formulation; anti-infectives

INTRODUCTION

Community-acquired pneumonia (CAP) is an inflammatory condition of the lungs due to an infection arising from sources external to a hospital or extended-care facility (e.g., nursing home) setting. Although CAP can be brought about by bacteria, viruses, or fungi, the most common cause of CAP in adults is still via a bacterial infection. The severity of CAP has been linked to the extent of inflammation in the body, as inflammation is the body's natural response to the invading infection-causing pathogens.^{1,2} Despite advances in vaccination and antimicrobial therapy, CAP remains as a leading cause of human morbidity and mortality, and has afflicted the world population with a huge socio-economic cost.^{1–4}

Chronic obstructive pulmonary disease (COPD) is a pulmonary condition where the airways become chronically inflamed and irreversibly narrowed, hence leading to a shortness of breath. Furthermore, patients with COPD appear to be at

a higher risk of developing CAP than patients in the general population.^{5,6} Recent studies have reported on the impaired immunological mechanisms in COPD patients brought about by the defective alveolar macrophage phagocytosis of bacteria. This consequently increases the susceptibility of COPD patients to infections.^{7,8} Moreover, COPD patients tend to develop persistent bacterial colonization in the lower airways through the inflammatory mechanism, hence leading to exacerbations of COPD and progression of airway obstruction.^{9,10} Therefore, CAP patients with COPD frequently experience poorer clinical outcomes than patients without COPD. The former has been reported to exhibit higher 30- and 90-day mortality than the latter.¹¹ As these risks tend to increase with COPD severity, it is vital for CAP patients with COPD to seek medical intervention early to prevent COPD exacerbations and limit disease progression.

Currently, antibiotic treatment remains the mainstay of CAP therapy. However, patients with obstructive airways disease complicated by CAP could also benefit from the inclusion of inhaled corticosteroids into their treatment regimen in view of the drug's potent immunomodulatory and anti-inflammatory properties.³ Although the release of inflammatory mediators from alveolar macrophages is useful for eliminating invading pathogens from pulmonary infections, excessive releases are on the contrary, harmful to the lungs. Hence, modulation of the

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inflammatory response is vital to creating a balance between the beneficial and harmful effects.^{12–14}

Corticosteroids are the most commonly used physiological inhibitors for inflammation. They interfere with the inflammatory expression and action that are associated with COPD and pneumonia. The inflammatory response in the lung is a complex process that involves the coordinated expression of both proinflammatory and anti-inflammatory cytokines.¹ Corticosteroids can switch off genes that encode proinflammatory cytokines such as tumor necrosis factor alpha, interleukin-1 beta, and interleukin-6, and switch on those that encode anti-inflammatory cytokines such as interleukin-10.^{15–17} For CAP patients with COPD, the downregulation of the unwanted excessive cytokine response by corticosteroids has the beneficial effect of reducing excessive pulmonary inflammation and accelerating clinical recovery. In addition, bronchospasms (wheezing), a common condition found in these patients, could also be treated with the corticosteroids.^{3,18,19}

Understandably, clinicians have therefore applied the use of corticosteroids as an adjunctive therapy (i.e., anti-inflammatory properties) for different infectious diseases as early as the 1950s.²⁰ The use of corticosteroids in combination with antibiotics has been proven to be effective and safe in several randomized controlled trials in patients with bacterial and tuberculous meningitis,^{21–23} tuberculous pericarditis,²⁴ septic arthritis,²⁵ and septic shock.²⁶ In CAP–COPD patients, coadministration of corticosteroids with antibiotics has been shown to be safe and efficacious, and is therefore frequently administered by the physician for treatment.^{2,3} On a further note, for smokers who are predisposed to COPD and bacterial infections (i.e., tobacco smoke compromise the antibacterial function of leukocytes, including neutrophils, monocytes, T cells, and B cells),⁶ treatment via the steroid–antibiotic combination will no doubt be a useful and timely therapeutic intervention as well. Unfortunately, no combined steroid–antibiotic dry powder formulation is available commercially or has been reported in the academic literature.

Hence, the objective of this study is to pioneer, via state-of-the-art nanospray drying,^{27,28} a novel inhalable dry powder formulation incorporating both an antibiotic and a corticosteroid for the treatment of bacterial infections in CAP–COPD patients. A dry powder inhaler (DPI) formulation is favored over the other aerosol delivery modes [e.g., nebulizer or metered dose inhaler (MDI)] because of the improved formulation stability associated with the powdered drug (as compared with the solution or suspension in the MDI and nebulizer), improved delivery efficiency, portability, ease-of-use, and the avoidance of undesired precipitation in solutions (e.g., in the nebulizer).^{29–32}

Ciprofloxacin hydrochloride (CIP) and beclomethasone dipropionate (BP) were selected as the model antibiotic and corticosteroid in view of their broad-spectrum activity against both gram-negative and gram-positive bacteria (especially in CAP⁴), and robust anti-inflammatory activity,³³ respectively. Among the members of the fluoroquinolone family (a class of broad-spectrum antibiotics), the antibiotic ciprofloxacin has been known for its clinical efficacy and low potential for adverse effects.^{34–36} In addition, the efficacy, safety, and tolerability of ciprofloxacin DPI in human subjects had been successfully evaluated under Phase I and II clinical trials.^{36,37} Phase III efficacy studies are currently underway.³⁸ Inhaled BP, being a robust anti-inflammatory agent, was previously demonstrated to be

Table 1. Spray-Drying Parameters

Parameters	
Spray mesh size (μm)	7
Feed concentration (w/v, %)	0.7
Cosolvent (water–methanol) ratio (v/v)	1:3.5
Nitrogen flow rate (L/min)	120
Relative spray rate (mL/h)	4
Inlet temperature (°C)	75
Outlet temperature (°C)	38–42
Yield (%)	80–90

an effective treatment for patients with nonasthmatic chronic airflow obstruction.³⁹

The feasibility of combining the antibiotic and the corticosteroid as an inhalable dry powder formulation for direct concomitant delivery to the lung was investigated. The spray-dried binary formulation that contained CIP and BP (SD-CIP/BP), as well as their single counterparts (SD-CIP and SD-BP), was evaluated for their physicochemical characteristics, aerosol performance, and antimicrobial properties.

MATERIALS AND METHODS

Materials

CIP and BP were supplied from Junda Pharmaceutical Company Ltd. (Changzhou, China). Disodium hydrogen phosphate and phosphoric acid were purchased from Sigma Chemical Company (St. Louis, Missouri). Ultrapure water was used in the experiments. High-performance liquid chromatography (HPLC) grade acetonitrile was supplied by Merck (Darmstadt, Germany). The model bacteria used in the study were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia) and included *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* obtained from the National University Hospital (Singapore). Mueller-Hinton broth (Oxoid, Basingstoke, UK) was used as the culture media for the antimicrobial activity test.

Preparation of Spray-Dried Particles

Powders of CIP (SD-CIP), BP (SD-BP), and binary combination powders of BP/CIP in a weight ratio of 1:32.5 (SD-CIP/BP) were prepared by spray drying CIP alone, BP alone, and CIP with BP from a methanol–water cosolvent feedstock using a B-90 Nano Spray Dryer (Büchi Labortechnik AG, Flawil, Switzerland)^{27,28} with operating parameters as detailed in Table 1. All solutions were filtered through a 0.45-μm syringe filter (Millipore, Bedford, Massachusetts) prior to spray drying to minimize blockage due to any undissolved particles at the spray mesh. The spray-dried powders were stored in a desiccator at room temperature for further characterization.

Surface Morphology

The morphology of the powder particles was examined by field emission scanning electron microscopy (FESEM; JEOL JSM-6700; JEOL; Tokyo, Japan) at 5 kV. Prior to imaging, the samples were dispersed onto carbon sticky tabs and coated with gold for 100 s using a sputter coater (Cressington 208HR; Cressington, Watford, UK).

Particle Size Analysis

The particle size distribution of the spray-dried powders was determined by laser diffraction using Malvern Mastersizer 2000 (Malvern Instruments, Malvern, UK) using the Scirocco dry dispersion unit. The powders were dispersed in triplicates at 3 bars of pressure using refractive index of 1.520 for SD-CIP and SD-BP/CIP, and 1.564 for SD-BP.

Powder Crystallinity

Powder crystallinity of the samples was assessed by powder X-ray diffraction at room temperature using an X-ray diffractometer (D8 Advance; Bruker AXS GmbH, Karlsruhe, Germany). Samples were scanned from 2° to 50° (2 θ) at an angular increment of 0.04° and at 1 s per step using Cu K α radiation generated at 35 kV and 40 mA.

Surface Composition Evaluation

The surface elemental composition of the powdered samples was measured using X-ray photoelectron spectroscopy (XPS) on a VG ESCALAB 250 spectrometer (Thermo Electron, East Grinstead, West Sussex, UK) that was equipped with a non-monochromatized Al α X-ray source (1486 eV). Measurements were recorded for a 20-eV pass energy, a 0.1-eV kinetic energy step, and a 0.05-s dwelling time. The powders were pressed onto double-sided conductive adhesive tapes. Experimental molar percentages of all elements except hydrogen were derived from the XPS peak areas as described elsewhere.⁴⁰ Elemental weight percentages (Table 4) were obtained via multiplying the molar percentages with the respective atomic mass.

In Vitro Aerosol Performance

The aerosol performance of the spray-dried powders was assessed using a multistage liquid impinger (MSLI; Copley Scientific, Nottingham, UK) coupled with a United State Pharmacopoeia (USP) stainless steel throat. The method followed the procedure specified for DPIs in the British Pharmacopoeia.⁴¹ Prior to testing, 20 mL of water–methanol cosolvent in the ratio 1:3.5 (v/v) was added across all four stages of the MSLI. Approximately 20 $\pm$ 2 mg of powder from each formulation was filled into a hydroxypropyl methylcellulose capsule (size 3; Capsugel®, Morristown, New Jersey), loaded into the high-efficiency Aerolizer® inhaler (Novartis Pharmaceuticals, Basel, Switzerland)⁴² and pierced. The powder was then dispersed for 4 s at 60 L/min and 2.4 s at 100 L/min. The flow through the MSLI was measured using a calibrated flow meter (TSI Model 4040C; TSI Instrument Ltd., Buckinghamshire, UK), controlled by a high-capacity vacuum pump (Model HCP5; Copley Scientific) and a critical flow controller (TPK 2000, Copley Scientific). After dispersion, the device, capsule, throat, and each stage of the MSLI were washed separately and thoroughly using water–methanol cosolvent. The solutions were then assayed by HPLC after appropriate sample dilutions. Each dispersion test was performed in triplicates to obtain mean values. Dispersion was carried out in a walk-in environmental chamber (Synergys, Singapore) maintained at 25°C and 40% relative humidity. In this study, fine particle fraction (FPF) represents the mass fraction of drug particles smaller than 5 μ m in the aerosol cloud relative to the total mass recovered and was obtained by interpolation to the cumulative percent undersize at 5 μ m. FPF(emitted) was obtained when the fine particle dose was expressed relative to the emitted dose.

Drug Content Quantification

Drug contents in the spray-dried powders and in the aliquots collected from the MSLI were analyzed via HPLC (1100 series; Agilent Technologies, Santa Clara, California). For CIP assays, a 20 μ L aliquot sample was injected into the HPLC system equipped with a Zorbax SB C-18 column (4.6 mm $\times$ 150 mm, 3.5 μ m) (Agilent Technologies) as the stationary phase (column temperature 25°C), and a mixture of 0.025 M disodium hydrogen phosphate buffer (adjusted to pH 3.0 with phosphoric acid) and acetonitrile (80:20, v/v) as the mobile phase, at a flow rate of 1 mL/min and an UV detection wavelength of 293 nm. For the assay of BP, the mobile phase consisted of acetonitrile–water (60:40, v/v) delivered at 1.5 mL/min under a UV detection wavelength of 250 nm. The retention time of CIP and BP was 2.3 and 4.7 min, respectively.

Powder Dissolution

Two-hundred milliliter dissolution medium (0.2%, w/v, sodium dodecyl sulfate solution) at 37.0 $\pm$ 0.5°C was passed through a 25-mm internal diameter flow-through cell (Millipore Swinnox filter holders, utilizing a Pall HT Tuffryn 0.2 μ m membrane disc filters (Pall Corporation, Port Washington, NY)) containing 20 mg of sample, and recirculated in a closed-loop configuration at 1.6 mL/min using a peristaltic pump (pump speed stability of $\pm$ 0.5%) (Gilson MiniPuls3; Gilson, Inc., Middleton, WI).^{43,44} At regular time intervals, 0.7 mL samples were withdrawn, diluted with 0.5 mL ethanol, and then analyzed for their concentrations using the previously described HPLC methods. Studies were carried out in triplicate.

Determination of Antimicrobial Minimum Inhibitory Concentration

The *in vitro* activity of SD-BP, SD-CIP, and SD-BP/CIP was tested against bacterial type strains of *K. pneumoniae* ATCC 12885, *P. aeruginosa* ATCC 90207, and *S. aureus* ATCC 4330. The microdilution test was performed in accordance to the Clinical and Laboratory Standards Institute (CLSI) procedures⁴⁵ using a final bacterial inoculum of approximately 5 $\times$ 10⁵ (colony forming units) CFU/mL in a sterile 96-well plate bearing twofold drug dilutions. The minimum inhibitory concentration (MIC) was defined as the lowest drug concentration that inhibited the visible growth of microorganisms after an overnight incubation. The MIC assays were carried out in duplicate for each strain.

Statistical Analysis

Statistical significance was carried out using one-way analysis of variance with Tukey's post-hoc analysis at a *p* value of 0.05 using Minitab.

RESULTS AND DISCUSSION

Preparation of Spray-Dried Particles

Spray-dried powders of corticosteroid alone (SD-BP), antibiotic alone (SD-CIP), and binary combination of corticosteroid and antibiotic (SD-BP/CIP) were successfully prepared and collected at yields of more than 80% (Table 1) using the Nano Spray Dryer B-90 (Büchi Labortechnik AG, Flawil, Switzerland).²⁸ High-product yield is readily achieved on the Nano Spray Dryer

Table 2. Composition of the Spray-Dried Particles

Formulation	Drug Content (w/w, %)				% of Ideal (Relative to Feed)	
	Feed		Actual			
	BP	CIP	BP	CIP	BP	CIP
SD-BP	100.0	–	99.7 ± 0.5	–	99.7 ± 0.5	–
SD-CIP	–	100.0	–	99.3 ± 0.7	–	99.3 ± 0.7
SD-BP/CIP	3.0	97.0	2.9 ± 0.1	97.7 ± 0.8	96.7 ± 3.3	100.7 ± 0.8

Drug contents measured by HPLC.

Mean ± SD ($n = 3$).

B-90, with published reports listing it as between 70% and 90%.^{27,46,47}

CIP is a water-soluble antibiotic, whereas BP is a poorly water-soluble steroid that can only be dissolved in organic solvents such as methanol, chloroform, or acetone. In order to cospray dry both hydrophilic and hydrophobic APIs simultaneously for the production of particles with uniform drug composition, cosolvent selection and ratio are both very important aspects. In this work, a water–methanol mix was selected as the cosolvent system for BP and CIP in view of their mutual miscibility and low toxicity. The water–methanol cosolvent volume ratio was adjusted to 1:3.5 to ensure good solubility of both APIs in the cosolvent system with no precipitation.

Unlike oral corticosteroids that must travel throughout the patient's body to reach the lung, inhaled corticosteroids are typically used in much smaller doses (i.e., a few hundred micrograms) as there is direct lung targeting.⁴⁸ Furthermore, the maximum daily dose of inhaled BP has been recommended to be at approximately 1 mg to avoid significant reduction in plasma cortisol concentrations.^{49,50}

Antibiotics are widely used to treat bacterial infections by either killing (bactericidal) or inhibiting the growth (bacteriostatic) of bacteria. It is usually used at high concentrations to ensure good tissue penetration and maximum bacterial kill.^{51,52} Currently, Cipro Inhale[®], an inhaled antibiotic dry powder formulation undergoing Phase III clinical trial, has been found to be safe and well tolerated (via Phase I and II trials), when applied at a single inhaled dose of 32.5 mg.^{36,37}

In this work, the binary dry powder formulation containing BP and CIP was prepared at 1:32.5 weight ratio (adapted from reported clinical dosing). Although the weight ratio of BP was as low as 3% in the binary formulation, it was still well preserved (as close as 100% in comparison to the feed, Table 2) in the final spray-dried powder.

Surface Morphology and Particle Size

The surface morphologies of the single and binary spray-dried particles are shown in Figure 1. All the spray-dried particles were generally spherical in geometry, with the SD-CIP having a smoother surface (Figs. 1c and 1d) than the SD-BP (i.e., slightly corrugated surface) (Figs. 1a and 1b). The surface morphology of SD-BP/CIP (Figs. 1e and 1f) was found to be closer to that of SD-BP, whereby a slightly corrugated surface was obtained. This observation was not surprising, even though CIP was the major component of the powder [i.e., 97%, (w/w) CIP vs. 3% (w/w) BP]. The final distribution of each solute component in the spray-dried particle would very much depend on the solutes' surface activities (which lead to the preferential adsorption of components on the sprayed droplet surface) and diffusivities

in the spray solution. In the present binary cosolvent system (i.e., methanol–water), the saturation of hydrophobic API (BP) occurred much faster than that of the hydrophilic API (CIP) because of the preferential evaporation of methanol over water and the associated changes in methanol–water ratio during the drying process. This then led to the accumulation and precipitation of the hydrophobic component at the liquid–air interface. Consequently, more hydrophobic component is found on the surface of the dried particles.^{53–55} Thus, this explains the similar surface morphologies of both SD-CIP/BP and SD-BP. The XPS results discussed under *Surface Composition Evaluation* further supports this finding.

As all the single and binary spray-dried powders were prepared under the same spray-drying conditions, they were thus found to have comparable particle size and span (Table 3). The particle size analysis revealed that all the spray-dried powders were within the respirable size range, with a d_{50} of 2.21 ± 0.07 , 2.36 ± 0.02 , and 2.36 ± 0.01 μm for SD-BP, SD-CIP, and SD-BP/CIP, respectively.

Powder Crystallinity

The diffractogram of the raw materials, single spray-dried powders, and cospray-dried powder is shown in Figure 2. The sharp peaks found in the diffractograms of Raw-CIP and Raw-BP indicated that both starting materials were crystalline powders. The X-ray diffraction pattern of SD-BP showed only a single diffused broad peak, indicating its amorphicity. In comparison, reduced crystallinity was observed for SD-CIP. This observation was not surprising as we have previously reported on the reduced crystallinity of SD-CIP on the Nano Spray Dryer B90.⁴⁶ The X-ray diffraction pattern of SD-BP/CIP appeared to be very similar to that of SD-CIP because of the high weight ratio of CIP in the formulation.

Surface Composition Evaluation

Table 4 illustrates the experimentally measured surface composition of the raw materials and spray-dried powders that was derived from the XPS peak areas. Generally, the experimentally measured surface elemental composition of the raw materials (i.e., Raw-BP and Raw-CIP) was comparable to their spray-dried counterparts (i.e., SD-BP and SD-CIP). This implies that the possibility of surface composition alteration via spray-drying process was minimal. On the contrary, the experimentally measured surface composition of the binary spray-dried powder, SD-BP/CIP, did not follow the trends of either SD-BP or SD-CIP. Hence, neither BP nor CIP solely resided on the surface of SD-BP/CIP. In order to further evaluate the surface composition of binary SD-BP/CIP, the expected elemental weight percentages of the binary SD-BP/CIP was calculated

Figure 1. Field-emission scanning electron microscopy images of spray-dried powders. (a) SD-BP, (b) SD-BP (close-up view), (c) SD-CIP, (d) SD-CIP (close-up view), (e) SD-BP/CIP, and (f) SD-BP/CIP (close-up view).

(by assuming that both BP and CIP were distributed evenly on the particle surface). The expected elemental weight percentages could be calculated from the elemental weight percentages that were measured experimentally on the raw materials (Table 5). For example, the expected elemental weight percentage of carbon in SD-BP/CIP was the summation of the products obtained via multiplying the weight percentages of carbon in Raw-BP with weight proportion of BP (i.e., 3%, w/w) in SD-BP/CIP and weight percentages of carbon in Raw-CIP with the weight proportion of CIP in SD-BP/CIP (i.e., 97%, w/w). The

experimentally measured elemental weight percentages of the binary SD-BP/CIP were then compared with the expected elemental weight percentages. Any difference between the weight percentages and expected elemental weight percentages suggested an over- or under-abundance of certain elements and hence the types of molecules on the particle surface. From Table 5, the expected elemental weight percentages of the binary SD-BP/CIP was comparable to the weight percentages of Raw-CIP and SD-CIP. It was as expected for an assumed even distribution of BP and CIP on the particle surface. BP being a

Table 3. Volume Particle Size Distribution of Spray-Dried Powder

	SD-BP (μm)	SD-CIP (μm)	SD-BP/CIP (μm)
d_{10}	1.01 ± 0.01	0.93 ± 0.01	0.88 ± 0.06
d_{50}	2.21 ± 0.07	2.36 ± 0.02	2.36 ± 0.01
d_{90}	4.48 ± 0.19	5.43 ± 0.29	5.37 ± 0.29
Span	1.57 ± 0.04	1.91 ± 0.13	1.91 ± 0.16

Mean $\pm$ SD ($n = 3$). d_{10} , volume diameter under which 10% of the sample resides. d_{50} , volume median diameter. d_{90} , volume diameter under which 90% of the sample resides.Span = $(d_{90} - d_{10})/d_{50}$.

very minor component (just 3% weight ratio), would be considered negligible when compared with the presence of CIP (97% weight ratio) on the surface.

Nitrogen and fluorine are two elements that are specific to CIP ($\text{C}_{17}\text{H}_{18}\text{FN}_3\text{O}_3 \cdot \text{HCl}$) but not BP ($\text{C}_{28}\text{H}_{37}\text{ClO}_7$), so a lower C/N, C/F, O/N, O/F, Cl/N, and Cl/F experimental ratio in SD-BP/CIP over that of SD-CIP could possibly suggest that CIP resided less preferentially on the surface of SD-BP/CIP as compared with BP (Table 6). However, because of the large weight ratio of CIP (97%) in SD-BP/CIP, some of the CIP molecules could still be found on the particle surface of SD-BP/CIP. The enrichment of BP on the particle surface could also be inferred from the C/O ratios. SD-BP has a higher C/O ratio than SD-CIP and the high C/O ratio obtained for SD-BP/CIP again implies that BP preferentially resides on the surface of the particle.

The enrichment of BP on the particle surface is an expected outcome, as during spray drying, the more volatile solvent (i.e., methanol) in a mixed methanol–water droplet would evaporate much faster than water. Due to the low solubility of BP in the remaining aqueous phase, precipitation of the compound onto the surface of the alcohol-deficient droplets and hence the dried particle, is hence encouraged. Similarly, Kumon et al.⁵³ had previously shown that hydrophobic drugs (rather than the hydrophilic excipient) tend to reside on the particle surface when spray dried from an alcohol–water mix.

Table 4. Surface Composition of the Raw Materials and Spray-Dried Powders

Sample	Element	Molar Percentages	Weight Percentages
Raw BP ($\text{C}_{28}\text{H}_{37}\text{ClO}_7$)	Carbon	73.9	60.5
	Oxygen	17.9	19.6
	Chlorine	8.2	19.9
Raw-CIP ($\text{C}_{17}\text{H}_{18}\text{FN}_3\text{O}_3 \cdot \text{HCl}$)	Carbon	63.9	49.7
	Oxygen	11.3	11.8
	Chlorine	10.7	24.7
	Nitrogen	10.7	9.7
	Fluorine	3.4	4.1
SD-BP ($\text{C}_{28}\text{H}_{37}\text{ClO}_7$)	Carbon	72.4	59.7
	Oxygen	20.1	22.1
	Chlorine	7.5	18.2
SD-CIP ($\text{C}_{17}\text{H}_{18}\text{FN}_3\text{O}_3 \cdot \text{HCl}$)	Carbon	64.0	52.1
	Oxygen	12.6	13.5
	Chlorine	8.6	20.3
	Nitrogen	10.5	9.8
	Fluorine	3.4	4.3
SD-BP/CIP ($\text{C}_{28}\text{H}_{37}\text{ClO}_7$) ($\text{C}_{17}\text{H}_{18}\text{FN}_3\text{O}_3 \cdot \text{HCl}$)	Carbon	53.6	42.4
	Oxygen	35.6	37.5
	Chlorine	7.0	16.3
	Nitrogen	3.0	2.8
	Fluorine	0.9	1.1

In Vitro Aerosol Performance

In this study, the aerosol performance of the spray-dried powders was studied under two levels of realistic inspiratory effort: low (60 L/min) and high (100 L/min). The lower airflow of 60 L/min is likely to be generated by patients with compromised lung functions (e.g., pneumonia⁴⁶), whereas the higher airflow of 100 L/min through the Aerolizer[®] inhaler represents a comfortable inspiratory effort readily achieved by patients with healthy lung functions.^{53,56,57}

Figure 2. X-ray diffraction patterns of Raw-CIP, Raw-BP, SD-BP, SD-CIP, and SD-BP/CIP.

Table 5. Calculation of Expected Elemental Percentage in SD-BP/CIP

Sample	Drug Component	Weight Proportion (%) ^a	Theoretical Elemental Weight Percentages ^b		Expected Elemental Weight Percentages ^c		Experimental Elemental Weight Percentages ^d	
SD-BP/CIP	BP	3	Carbon	1.8	Carbon	50.0	Carbon	42.4
			Oxygen	0.6	Oxygen	12.0	Oxygen	37.5
			Chlorine	0.6	Nitrogen	9.4	Nitrogen	2.7
	CIP	97			Chlorine	24.6	Chlorine	16.3
			Carbon	48.2	Fluorine	4.0	Fluorine	1.1
			Oxygen	11.4	Total	100	Total	100
			Nitrogen	9.4				
			Chlorine	24.0				
			Fluorine	4.0				

^a [Weight ratio of BP or CIP in feed/(total weight ratio in feed)] × 100; weight ratio of BP–CIP in feed is 1:32.5.

^b Weight percentages (raw materials) × weight proportion.

^c Summation of theoretical elemental weight percentages (e.g., for carbon, expected elemental weight percentages is 1.8 + 48.2 = 50.0), assuming that both BP and CIP were distributed evenly on particle surface.

^d Molar percentages × atomic weight of the element.

Table 6. Various Elemental Ratios of Spray-Dried Powders

Ratio	SD-BP	SD-CIP	SD-BP/CIP
Carbon/oxygen	1:0.37	1:0.26	1:0.88
Carbon/nitrogen	–	1:0.19	1:0.07
Carbon/fluorine	–	1:0.08	1:0.03
Oxygen/nitrogen	–	1:0.73	1:0.07
Oxygen/fluorine	–	1:0.32	1:0.03
Chlorine/nitrogen	–	1:0.48	1:0.17
Chlorine/fluorine	–	1:0.21	1:0.07

The aerosol performance results of the spray-dried powders are shown in Figure 3 and Table 7. Generally, SD-CIP was found to perform poorer, with FPFs of approximately 17.8% at 60 L/min and approximately 25.1% at 100 L/min as compared with SD-BP that had FPFs of approximately 32.0% at 60 L/min and approximately 41.7% at 100 L/min. This might be due to SD-CIP's higher agglomeration propensity in the aerosol (evident via the high powder deposition throughput on Stage 1) (Fig. 3b). However, agglomerate break-up was achievable when the flow rate was increased from 60 to 100 L/min (Fig. 3e). For SD-CIP, powder deposition on Stage 1 decreased from approximately 24% to approximately 19.5% with an increase in flow rate. In addition, increasing the flow rate from 60 to 100 L/min also has the advantage of improving the emitted fraction [i.e., inferred via an improved FPF but similar FPF (emitted)] (Table 7).

Interestingly, the MSLI deposition profile of binary SD-BP/CIP followed that of SD-BP rather than SD-CIP, even though SD-CIP was the major component in the formulation (weight ratio of 1:32). Possibly, this could be because of the distribution of BP on the particle surface of SD-BP/CIP, which in a way reinforces the earlier findings from the XPS and scanning electron microscopy analyses. For the binary SD-BP/CIP, there was concomitant deposition observed across all stages (including the throat, inhaler, and capsule) at 60 and 100 L/min, suggesting uniformity in the powder and/or aerosol (Figs. 3c and 3f). From Table 7, it also does seem that formulating the drugs into a binary mix has the advantage of improving their delivery more effectively to the patient. FPF and FPF(emitted) for SD-BP and SD-CIP were generally much lower than BP and CIP in SD-BP/CIP. For CIP in particular, the improvement was significant when it was codelivered with BP at 60 and 100 L/min ($p < 0.05$).

In Vitro Drug Release

BP, being a Biopharmaceutical Classification System class II drug (i.e., low solubility and high permeability) is limited by dissolution-rate-limited kinetics.⁵⁸ Therefore, any improvements to the drug's pulmonary solubility would be beneficial to the absorption process in the lungs.⁵⁹

Figure 4 shows that when BP (a poorly water-soluble drug) was formulated into a binary mix with the water-soluble CIP, its dissolution rate and extent were drastically improved over the single species ($p < 0.05$). Hence, this formulation approach does seem to be highly effective in “lifting” the performance of the poorer-performing species to almost the same extent as the robust species. Drug release of CIP in the binary mix was comparable to that of the single species. The release mechanism in this case was analogous to an “API–API solid dispersion system,” whereby the poorly water-soluble BP was dispersed into the more water-soluble CIP matrix, hence enhancing the wettability and solubility of the former (Fig. 5).⁶⁰

Antibacterial Activity of Spray-Dried Powders

K. pneumoniae, *P. aeruginosa*, and *S. aureus* are common etiologic agents for CAP.⁴ Table 8 shows the antibacterial activity of the spray-dried powders with MICs of SD-BP, SD-CIP, and SD-BP/CIP determined via the microdilution method on type strains of *K. pneumoniae*, *P. aeruginosa*, and *S. aureus*. As expected, the MIC of SD-BP was not determinable as BP is a typical corticosteroid that does not exhibit any antibacterial properties. The MICs of SD-BP/CIP on the various bacterial species were found to be comparable to the MICs of SD-CIP, suggesting the antibacterial properties of CIP in the binary mix was adequately retained.

CONCLUSIONS

A novel steroid–antibiotic DPI formulation (SD-CIP/BP) was successfully pioneered via nanospray drying. The powder obtained was of respirable size (d_{50} of $\sim 2.3 \mu\text{m}$), partially crystalline (potentially improved storage stability), and had BP preferentially deposited on the particle surfaces. When dispersed via an Aerolizer at 60 and 100 L/min, the powder showed concomitant and uniform *in vitro* deposition profile across all impaction stages, with obtained FPFs of

Figure 3. *In vitro* deposition of (a) SD-BP, (b) SD-CIP, (c) SD-BP/CIP at 60 L/min and (d) SD-BP, (e) SD-CIP, and (f) SD-BP/CIP at 100 L/min. Data presented as mean $\pm$ SD ($n = 3$). S1–S4 denote impactor stages, followed by their corresponding lower aerodynamic cutoff diameter in parentheses.

Table 7. Deposition Parameters (Mean $\pm$ SD, $n = 3$) of Different Formulations Measured by MSLI at 60 and 100 L/min

	60 L/min				100 L/min			
	SD-BP	SD-CIP	SD-BP/CIP		SD-BP	SD-CIP	SD-BP/CIP	
			BP	CIP			BP	CIP
FPF ^a (%)	32.0 $\pm$ 1.2	17.8 $\pm$ 1.0	36.4 $\pm$ 1.3	38.3 $\pm$ 0.8*	41.7 $\pm$ 0.3	25.1 $\pm$ 4.0	44.5 $\pm$ 2.1	45.9 $\pm$ 1.5*
FPF (emitted) ^b (%)	47.6 $\pm$ 1.2	21.6 $\pm$ 2.0	49.0 $\pm$ 2.5	51.3 $\pm$ 1.9	48.5 $\pm$ 0.2	33.8 $\pm$ 3.7	50.0 $\pm$ 0.7	50.6 $\pm$ 0.2

^aFPF - fine particle fraction

^bFPF (emitted) - emitted fine particle fraction

*Significant difference from CIP in SD-CIP ($p < 0.05$)

Figure 4. *In vitro* dissolution rate profiles of CIP and BP in individual and combined formulations.

Figure 5. Schematic diagram illustrating release mechanism.

Table 8. Minimum Inhibitory Concentration Values of the Spray-Dried Powders

Bacteria Species and Strain	MIC ($\mu\text{g/mL}$)		
	SD-BP	SD-CIP	SD-BP/CIP
<i>Klebsiella pneumoniae</i> ATCC 12885	ND ^a	1–2	2
<i>Pseudomonas aeruginosa</i> ATCC 90207	ND	2	1
<i>Staphylococcus aureus</i> ATCC 4330	ND	1–2	2

^aND, not determined because of the absence of inhibition.

approximately 36%–46%. Favorably, when formulated as a binary mix, both CIP and BP showed much higher drug release and FPFs over their singly delivered counterparts, and was also active against the respiratory tract infection-causing bacteria *K. pneumoniae*, *P. aeruginosa*, and *S. aureus*. Cospray-dried powder of BP and CIP (SD-BP/CIP) may hence be a novel and useful anti-inflammatory and antimicrobial formulation for CAP patients with COPD complications.

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A novel inhaled multi-pronged attack against respiratory bacteria

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ABSTRACT

Airway mucus hypersecretion is a common clinical feature of many severe respiratory diseases, and when complicated by a recalcitrant bacterial infection, the whole treatment regimen thereby becomes more challenging and protracted. The accumulation of thickened mucus secretions in the lower airways provides a nutrient-rich breeding ground for bacteria that promotes their growth and limits the ease of effective eradication. Unfortunately, no direct-inhaled dry powder formulation to treat these respiratory mucoid infections more effectively is available commercially. This work therefore seeks to develop a highly-efficacious ternary dry powder inhaler (DPI) formulation (ciprofloxacin hydrochloride (CIP), gatifloxacin hydrochloride (GAT) and ambroxol hydrochloride (AMB)) capable of delivering a novel multi-pronged attack (synergy, quorum quenching and mucociliary clearance) on *Pseudomonas aeruginosa*, a common respiratory bacteria found in mucoid infections. The powders were prepared via spray drying, evaluated on their aerosol performance via a multi-stage liquid impinger (MSLI) and tested for their efficacies in bacteria-spiked artificial sputum medium (ASM). The optimized particles were of respirable-size (d_{50} of $1.61 \pm 0.03 \mu\text{m}$) and slightly corrugated. When dispersed via an Aerolizer® inhaler at 60 L/min, the powder showed concomitant *in vitro* deposition, minimal capsule, device and throat retention, and highly promising and uniform fine particle fractions (of the loaded dose) of ~64–69%, which was a vast improvement over the singly-delivered actives. Favourably, when tested on bacteria-spiked ASM, the optimized ternary formulation (with AMB) was more effective at killing bacteria (i.e. faster rate of killing) than just the synergistic antibiotics alone (binary formulation; without AMB). In conclusion, a ternary antibiotic–(non-antibiotic) DPI formulation involving a unique multi-pronged attack mechanism was successfully pioneered and optimized for mucoid infections.

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1. Introduction

Airway mucus hypersecretion and a recalcitrant bacterial infection are the hallmarks of cystic fibrosis (CF), and as of today, this condition is the most common life-threatening inherited disease afflicting the Caucasian population (O'Sullivan and Freedman, 2009). The CF disorder is caused by mutations in a single gene on chromosome 7 that encodes the cystic fibrosis transmembrane conductance regulator (CFTR), a 1480 amino acid polypeptide

which regulates the transport of chloride ions across epithelial membranes (Tsui, 1995). Abnormalities of CFTR function result in low volume abnormal secretions leading to mucus plugs, impaired mucociliary clearance and eventually, chronic lung inflammation and infection (Bilton, 2008).

As CF patients with *Pseudomonas aeruginosa* infections typically have more rapid declines in lung function than non-*P. aeruginosa* infected individuals (Bilton, 2008), anti-pseudomonal therapy for this group of patients should hence be initiated early and aggressively upon first isolation of the bacteria, in order to prevent its evolution into a chronic infection (Valerius et al., 1991). Early eradication of *P. aeruginosa* infection is extremely crucial as initial *P. aeruginosa* colonization typically involves only non-mucoid strains that are highly susceptible to antibiotic agents. Failure to eradicate this initial colonization would allow the non-mucoid

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strains to freely mutate into the alginate-coated mucoid strains, which are more resistant to antibiotic agents. Resistance is brought about as a consequence of poor penetration of the antibiotic agents into the anaerobic sputum plugs (Oliver et al., 2000; Riordan et al., 1989; Worlitzsch et al., 2002).

Currently, antibiotic therapy remains the mainstay of treatment as gene or protein replacement therapies have yet to become commercial realities (Ratjen and Doring, 2003). Antibiotic treatment is aimed at reducing the bacterial load and inflammatory response in the lung, so as to ameliorate infective exacerbations and prevent or delay progressive respiratory failure.

Combination drugs, as innovations in pharmacotherapy (Wertheimer and Morrison, 2002), are increasingly used to combat the spread of antibiotic-resistant bacterial pathogens (Chait et al., 2007; Mouton, 1999; Wertheimer and Morrison, 2002). A combination of antibiotics provides a much broader spectrum of coverage than any single antibiotic alone, as the probability of a bacterium developing resistance to all the antibiotics employed in the combination therapy is much lower than in the case for an antibiotic in monotherapy (Lorian, 2005). Hence, this treatment approach is considered useful in the prevention and treatment of resistant bacterial infections (Chernish and Aaron, 2003; Doring et al., 2000; Mouton, 1999; Watkins et al., 1988; Weiss and Lapointe, 1995), and whenever a favorable synergistic effect is achieved, the whole treatment regimen thereby becomes more valuable, as the same killing effect could be obtained at much reduced doses and with fewer side effects (Chernish and Aaron, 2003). Although antibiotic combinations are commonly engineered in medicine to broaden the antimicrobial spectrum and generate synergistic effects, combinations of antibiotics with non-antibiotic drugs are increasingly gaining popularity as well (Ejim et al., 2011; Heaf et al., 1983; Kalan and Wright, 2011; Lee et al., 2014). These non-antibiotic drugs could potentially augment the activity of the antibiotic/s to bring about a more favorable outcome to the patient.

Mucus hypersecretion (increased mucus secretion) is a hallmark of CF whereby the rate of mucus secretion far exceeds the rate of removal by normal ciliary action (Balsamo et al., 2010). The accumulation of thickened mucus secretions in the lower airway of patients with CF provides a nutrient-rich breeding ground for bacteria that promotes their growth and limits the ease of effective eradication.

Hence, this work aims to explore a useful inhaled antibiotic–(non-antibiotic) formulation that comprises the use of a mucoactive agent with the antibiotic/s to facilitate simultaneous treatment and clearance of respiratory bacteria and mucus respectively (mucoactive agents are drugs used to alter the viscoelastic properties of mucus and promote secretion clearance (Balsamo et al., 2010)). A dry powder inhaler (DPI) formulation is preferred over the other aerosol delivery modes (e.g. nebulizer or metered dose inhaler (MDI)) in view of the improved formulation stability associated with the powdered drug (as compared with the solution or suspension in the MDI and nebulizer), improved delivery efficiency, ease-of-use, portability and the avoidance of undesired precipitation in solutions (e.g. in the nebulizer) (Coates et al., 2001; Elmore et al., 1996; Loughlin and Eigen, 1994; Traini and Young, 2009). Though largely popular, it is worthwhile to note some potential manageable side effects associated with the use of these inhalers and they include coughing, bronchospasm and hoarseness, with coughing probably being the most common local side effect.

To ensure maximum kill and effective penetration of antibiotics through the thick mucus, a novel multi-pronged strategy involving a combined synergy, quorum quenching and mucociliary clearance approach is proposed. Quorum sensing is the mechanism employed by pathogenic bacteria to mount successful infections by allowing them to regulate expression of gene networks that

co-ordinate virulence, antibiotic resistance and fitness responses. The process is mediated by small diffusible signaling molecules (Deep et al., 2011). Interference of the quorum sensing system by quorum quenching is recognized as a potential strategy for disease control (Dong et al., 2007; Tillotson and Theriault, 2013). Hence, in this study, antibiotic synergism (i.e. combined activity is more than the sum of individual activities) is expected to enhance the bactericidal activity (Chait et al., 2007; Lee et al., 2013), quorum quenching to disrupt bacterial cell-to-cell communication essential for infection and survival (Dong et al., 2007), and mucociliary clearance to remove/reduce the mucus layer for effective targeting of underlying bacteria (Balsamo et al., 2010). As airway mucus hypersecretion is a common clinical feature of many severe respiratory diseases like asthma, chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF) (Balsamo et al., 2010; Rogers, 2007), the proposed multi-pronged treatment strategy is thus expected to be highly applicable and beneficial to a diverse group of patients (e.g. pneumonia-COPD patients) (Lee et al., 2014).

In this work, ciprofloxacin hydrochloride (CIP) and gatifloxacin hydrochloride (GAT) were selected as the synergistic antibiotic pair (Lee et al., 2013), while ambroxol hydrochloride (AMB) was chosen for its multi-faceted properties (Paleari et al., 2011) that include quorum quenching (Dong et al., 2007; Hassett et al., 2002; Lu et al., 2010), mucoactivity (Paleari et al., 2011) and pulmonary protective effects (Li et al., 2012). In addition, ambroxol could potentially increase the bioavailability of antibiotics in the lung, particularly in the alveolar region (Paganin et al., 1995). This effect had been demonstrated for the fluoroquinolones (Paganin et al., 1995) and other drugs (Tewes et al., 2013), and similar benefits may be extended to the antibiotic–mucoactive combination.

2. Materials and methods

2.1. Materials

Ciprofloxacin hydrochloride (CIP), gatifloxacin hydrochloride (GAT) and ambroxol hydrochloride (AMB) were supplied from Jun-da Pharmaceutical Co. Ltd. (Changzhou, China). L-Leucine (LEU), disodium hydrogen phosphate, phosphoric acid, mucin, egg yolk emulsion, deoxyribonucleic acid (DNA) from fish sperm, casein hydrolysate, diethylenetriamine pentaacetic acid (DTPA), NaCl, KCl and mannitol were purchased from Sigma Chemical Co. (Louis, MO, USA). HPLC grade acetonitrile and molecular biology grade of 1 M Tris buffer (pH 8.0) were obtained from Merck (Darmstadt, Germany). American Type Culture Collection (ATCC) *P. aeruginosa* 90207 was used as the test microorganism.

2.2. Preparation of spray-dried powder

Powders of ciprofloxacin hydrochloride (SD-CIP), gatifloxacin hydrochloride (SD-GAT), ambroxol hydrochloride (SD-AMB), ternary combination powders of ciprofloxacin hydrochloride/gatifloxacin hydrochloride/ambroxol hydrochloride with (SD-CIP/GAT/AMB/LEU) and without leucine (SD-CIP/GAT/AMB), SD-CIP/GAT and SD-AMB/LEU were obtained by spray drying their aqueous solutions on a B-290 Mini Spray Dryer (Büchi Labortechnik AG, Flawil, Switzerland) with the following operating parameters: inlet temperature of 190 °C (outlet temperature 100 °C), atomization rate of 670 L/h, aspiration rate of 57.6 m³/h and feed rate of 3.5 mL/min. The feed solution concentration was maintained at 0.8% (w/v) for all the formulations. The ratio of CIP:GAT:AMB in the ternary formulations (both with and without leucine) was 1:2.5:5.5 (Lee et al., 2013). For SD-CIP/GAT/AMB/LEU and SD-AMB/LEU, the leucine content was 13% (w/w). The ratio of CIP:GAT for SD-CIP/GAT was maintained at 1:2.5. The spray-dried powders

were stored in a desiccator at room temperature for further characterization.

2.3. Surface morphology

The morphology of the powder particles was examined by field emission scanning electron microscopy (FESEM; JEOL JSM-6700; JEOL; Tokyo, Japan) at 5 kV. Prior to imaging, the samples were dispersed onto carbon sticky tabs and coated with gold for 100 s using a sputter coater (Cressington 208HR; Cressington, Watford, UK).

2.4. Particle size analysis

The particle size distribution of the spray-dried powders was determined by laser diffraction on the Malvern Mastersizer 2000 (Malvern Instruments, UK) using the Scirocco dry dispersion unit. The powders were scanned in triplicates at 3 bars of pressure using refractive index (RI) of 1.520.

2.5. Powder crystallinity

Powder crystallinity of the samples was assessed by powder X-ray diffraction (pXRD) at room temperature using an X-ray diffractometer (D8 Advance; Bruker AXS GmbH, Karlsruhe, Germany). Samples were scanned from 2° to 50° (2 θ) with an angular increment of 0.04° and at 1 s per step using Cu K α radiation generated at 35 kV and 40 mA.

2.6. In vitro aerosol performance

The aerosol performance of the spray-dried powders was assessed using a multi-stage liquid impinger (MSLI, Copley Scientific, Nottingham, UK) coupled with a United State Pharmacopoeia (USP) stainless steel throat in a walk-in environmental chamber (SynerSys, Singapore) maintained at 25 °C and 40% RH. Prior to testing, 20 mL of deionized water was added across all four stages of the MSLI. Approximately 20 $\pm$ 2 mg of powder from each formulation was filled into a hydroxypropyl methylcellulose (HPMC) capsule (size 3, Capsugel®, New Jersey, USA), loaded into the high efficiency Aerolizer® inhaler (Novartis Pharmaceuticals, Basel, Switzerland) (Heng et al., 2013) and pierced. The powder was then dispersed for 4 s at 60 L/min. The flow through the MSLI was measured using a calibrated flow meter (TSI Model 4040C, TSI Instrument Ltd., Buckinghamshire, UK), controlled by a high capacity vacuum pump (Model HCP5, Copley Scientific, Nottingham, UK) and a critical flow controller (TPK 2000, Copley Scientific, Nottingham, UK). After dispersion, the device, capsule, throat and each stage of the MSLI were washed separately and thoroughly using deionized water. The solutions were then assayed by high performance liquid chromatography (HPLC) after appropriate sample dilutions using a method modified from Al-Dgither et al. (2006) and Lee et al. (2013). Briefly, a 20 μ L aliquot sample was injected into the HPLC system equipped with a Zorbax SB-C18 column (4.6 mm $\times$ 150 mm, 3.5 μ m) (Agilent Technologies, California, USA) as the stationary phase (column temperature 30 °C), and a mixture of 0.025 M disodium hydrogen phosphate buffer (adjusted to pH 3.0 with phosphoric acid) and acetonitrile (80:20, v/v) as the mobile phase. A flow rate of 1.0 mL/min and an UV absorbance wavelength of 245 nm were employed for the simultaneous detection of ciprofloxacin hydrochloride, gatifloxacin hydrochloride and ambroxol hydrochloride at retention times of 3.1, 5.1 min and 10.1 min, respectively. In this study, fine particle fraction (FPF) represents the mass fraction of drug particles smaller than 5 μ m in the aerosol cloud relative to the total mass recovered and was obtained by interpolation to the cumulative percent undersize at 5 μ m. FPF(emitted) was obtained when the fine particle dose was

expressed relative to the emitted dose. At a flow rate of 60 L/min, the aerodynamic cut-off diameters of stages 1, 2, 3 and 4 are 13.0, 6.8, 3.1 and 1.7 μ m. Particles with diameter size less than 1.7 μ m were captured on an integral glass fiber filter paper (Pall Corporation, New York, USA). The mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) were calculated from the log-probability plot of the MSLI results. MMAD is the equivalent aerodynamic diameter at 50% cumulative mass of particles and GSD is the ratio of aerodynamic diameters at 84% and 50% cumulative mass.

2.7. Preparation of artificial sputum medium (ASM)

The artificial sputum medium comprising largely of mucin, DNA, amino acids and salts was prepared as according to Haley et al. (2012) with modifications to sterilizing the medium. Briefly, a solution of fish sperm DNA, salts (NaCl and KCl), casein hydrolysate and DTPA adjusted to pH 6.8 was filter sterilized through a 0.22 μ m membrane filter. Mucin solution prepared separately was adjusted to pH 6.8, autoclaved, then combined with the fish sperm DNA, casein hydrolysate and salt solution. Finally, sterile egg yolk emulsion was added to the entire contents thus making up the complete artificial sputum medium.

2.8. Preparation of *P. aeruginosa* spiked media

P. aeruginosa spiked ASM was prepared as follows: overnight cultures of *P. aeruginosa* ATCC 90207 were diluted in Mueller Hinton Broth (MHB) to an optical density measured at wavelength 600 nm (OD₆₀₀) of 0.01. This suspension was then further diluted 1:100 in fresh ASM media for use in antibacterial assays.

2.9. Antibacterial effect of SD-CIP/GAT/AMB/LEU spray-dried powder

The efficacy of the SD-CIP/GAT/AMB/LEU drug combination against *P. aeruginosa* ATCC 90207 was tested in the ASM media (benchmarked against SD-CIP/GAT and SD-AMB/LEU controls). Drug concentrations for testing started at 2/5.2/11.4 μ g/ml (CIP/GAT/AMB) and twofold dilutions of this were made in ASM media till a final concentration of 0.0039/0.010/0.022 μ g/ml was reached. The experiments were set up in 24 well plates using a total of 1 ml of *P. aeruginosa* spiked media per well. The drug combination was incubated with spiked media at 37 °C for 24 h after which CFU counts were performed. The experiment was carried out in triplicates.

3. Results and discussions

3.1. Surface morphology and particle size

The surface morphologies of the raw materials and spray-dried powders are shown in Fig. 1. While the Raw-CIP, Raw-GAT and Raw-AMB particles appeared as irregularly-shaped chips and chunks, the spray-dried particles were generally spherical in geometry. For the spray-dried combination particles, SD-CIP/GAT/AMB/LEU (Fig. 1h) had a slightly corrugated surface, whereas SD-CIP/GAT/AMB (Fig. 1g) was smoother in surface morphology. This difference could be due to the presence of the anti-adherent, leucine, which altered the surface properties of the former (Chew et al., 2005a). The particle size distribution of the formulations is summarized in Table 1. The particle size analysis revealed that all the spray-dried powders had d₅₀ ranging from 1.11 $\pm$ 0.05 to 2.08 $\pm$ 0.07 μ m.

Fig. 1. FESEM images of raw materials and spray-dried powders. (a) Raw-CIP, (b) Raw-GAT, (c) Raw-AMB, (d) SD-CIP, (e) SD-GAT, (f) SD-AMB, (g) SD-CIP/GAT/AMB and (h) SD-CIP/GAT/AMB/LEU.

3.2. Powder crystallinity

The diffractogram of the raw materials, single spray-dried and cospray-dried powders is shown in Fig. 2. The sharp peaks found in the diffractograms of Raw-CIP, Raw-GAT and Raw-AMB indicated that the starting materials were crystalline powders. While SD-CIP and SD-GAT powders were largely amorphous, SD-AMB exhibited reduced crystallinity. SD-CIP/GAT/AMB and SD-CIP/GAT/AMB/LEU powders were amorphous.

3.3. In vitro aerosol performance

The aerosolization performance results of the spray-dried powders are illustrated in Fig. 3 and Table 2. In this work, airflow of 60 L/min was used to simulate the reduced inspiratory effort achieved by patients with compromised lung functions (e.g. cystic fibrosis and pneumonia) (Lee et al., 2013, 2014). Generally, when the spray-dried powders were delivered individually (as single component systems), their FPFs ranged from being poor to margin-

Table 1

Volume particle size distribution of spray dried powder.

	SD-CIP (μm)	SD-GAT (μm)	SD-AMB (μm)	SD-CIP/GAT/AMB (μm)	SD-CIP/GAT/AMB/LEU (μm)
d_{10}	1.03 ± 0.02	1.10 ± 0.01	0.59 ± 0.05	0.54 ± 0.05	0.76 ± 0.02
d_{50}	2.02 ± 0.04	2.08 ± 0.07	1.41 ± 0.05	1.11 ± 0.05	1.61 ± 0.03
d_{90}	3.99 ± 0.24	4.00 ± 0.19	3.09 ± 0.10	2.15 ± 0.05	3.26 ± 0.13
Span	1.46 ± 0.11	1.39 ± 0.05	1.79 ± 0.14	1.45 ± 0.15	1.55 ± 0.07

Mean $\pm$ standard deviation, $n = 3$. d_{10} – volume diameter under which 10% of the sample resides. d_{50} – volume median diameter. d_{90} – volume diameter under which 90% of the sample resides.Span = $(d_{90} - d_{10})/d_{50}$. **Fig. 2.** XRD patterns of Raw-CIP, Raw-GAT, Raw-AMB, SD-CIP, SD-GAT, SD-AMB, SD-CIP/GAT/AMB and SD-CIP/GAT/AMB/LEU.

ally acceptable. In particular, SD-AMB exhibited a very low FPF of 8.6%, which was not surprising, as AMB is known to be highly hygroscopic and cohesive (an FPF value that is significantly higher than 30% is an index of good aerosol performance (Heng et al., 2012; Lee et al., 2013)). Device retention for all three compounds was substantial (i.e. ≈ 40 –60%) which also reflected on the cohesiveness of the individual spray-dried powders. Favorably, when formulated as a ternary mix, this retention decreased to under 30% (SD-CIP/GAT/AMB, Fig. 3d), and with further addition of the anti-adherent LEU, the retention amount dipped to under 15% (SD-CIP/GAT/AMB/LEU, Fig. 3e). Capsule retention for the latter was also found to be negligible (Fig. 3e). Generally, for the combination particles which had become single entities after co-spray drying, concomitant deposition was observed across the stages (including the throat, inhaler and capsule), with the exception of the lower stages (stages 3 to filter), where particle breakup probably contributed to the slight deviation in concomitancy. A possible explanation might be due to the impaction of the combination particles at the throat and the early stages of the MSLL that resulted in the release of particle fragments which were then captured downstream. These particle fragments might contain a higher proportion of one drug over the others, thus resulting in the slight deviation in concomitancy for the downstream stages.

From Table 2, it can be seen that the FPF of AMB could be improved when it was formulated as a ternary mix with the other drugs and excipient. For SD-CIP/GAT/AMB, the FPF was lifted from 8.6% to 14.7%, and in the presence of LEU (i.e. SD-CIP/GAT/AMB/LEU), it improved significantly to 64.7%. Favorably, for the optimized formulation with leucine, the FPF of CIP, GAT and AMB were lifted from low-to-moderate values of 24.1%, 17.2% and 8.6% respectively in the individuals, to more uniform and optimal values of 69.2%, 65.8% and 64.7% respectively. Furthermore, there was also an improvement to the emitted fraction (i.e. inferred via closely

similar FPF and FPF (emitted)). Therefore, co-delivery does seem to be highly beneficial in improving the dispersibility of the actives. SD-CIP/GAT/AMB/LEU was aerodynamically more superior than SD-CIP/GAT/AMB as the anti-adherent LEU had the beneficial effect of altering the surface properties of the particles (Chew et al., 2005a) to afford a slightly collapsed/corrugated spray-dried morphology, which favorably helped to improve the dispersive behavior (Fig. 1h) (Chew and Chan, 2001; Chew et al., 2005b). Indeed, particle morphology has long been known to be an important formulation factor influencing robust aerosol deposition (Crowder et al., 2002; Lee et al., 2011). In contrast to SD-CIP/GAT/AMB and the single component systems, the MMAD of SD-CIP/GAT/AMB/LEU was of the same order of magnitude as its d_{50} , and within the 1–5 μm aerodynamic diameter range, thus indicating its robustness in aerodynamic performance (Table 2). The MMAD values of SD-CIP/GAT/AMB and of the single component systems were much higher than their d_{50} , due to their higher propensity for particle aggregation (formulations lacking anti-adherent LEU) which resulted in much ‘larger particles’ which were mainly captured at the upstream stages (e.g. capsule, device, throat, stage 1). As the GSD, span and d_{50} were similar for SD-CIP/GAT/AMB/LEU and SD-CIP/GAT/AMB, the improvements to the aerodynamic qualities of the former could potentially be attributed mainly to the change in morphology rather than particle size.

3.4. Antibacterial activity of spray-dried powder

Fig. 4a shows the dose response kill curve of SD-CIP/GAT and the optimized SD-CIP/GAT/AMB/LEU compounds in artificial sputum medium (ASM) spiked with *P. aeruginosa*. Indeed, with the addition of AMB into the formulation, the bacterial killing effect was improved. The rate of $\log_{10}$ CFU decrease with antibiotic concentration was much faster for SD-CIP/GAT/AMB/LEU than SD-CIP/GAT, which suggested the presence of a ‘barrier-free’ environment that promoted strong antibiotic-bacteria interactions (i.e. breaking down the bacterium’s ‘shield’). For SD-CIP/GAT/AMB/LEU, full eradication of bacteria (i.e. $\log_{10}$ CFU = 0) was achieved at approximately half the antibiotic concentration required for SD-CIP/GAT (i.e. ≈ 0.25 and 0.45 $\mu\text{g}/\text{ml}$ respectively). This is indeed highly beneficial as it would mean that the effective penetration of antibiotics to the underlying bacteria could potentially result in lesser antibiotics needed for the same kill. This would have positive implications in terms of antibiotic dosage reduction, minimization of toxic side effects and the risk of encountering antibiotic resistance (due to the excessive use of valuable antibiotics). At present, the use (overuse, misuse and underuse) of antibiotics is driving the emergence of antimicrobial resistance in bacterial pathogens (Davies and Davies, 2010; Lister et al., 2009). Therefore, improved killing at much lower drug doses, coupled with the ‘portability’ and ‘inhaled’ nature of the formulation (i.e. dry powder inhalers) could potentially improve treatment outcomes and accelerate patient recoveries.

Fig. 3. In vitro deposition of spray-dried powders at 60 L/min. (a) SD-CIP, (b) SD-GAT, (c) SD-AMB, (d) SD-CIP/GAT/AMB and (e) SD-CIP/GAT/AMB/LEU. S1–S4 denote impactor stages, followed by their corresponding aerodynamic cut-off diameter in parentheses.

Table 2

Deposition parameters of different formulations measured by MSLI at 60 L/min.

	SD-CIP	SD-GAT	SD-AMB	SD-CIP/GAT/AMB			SD-CIP/GAT/AMB/LEU		
				CIP	GAT	AMB	CIP	GAT	AMB
FPF ^a (%)	24.1 ± 0.5	17.2 ± 0.6	8.6 ± 0.1	21.2 ± 0.6	16.2 ± 0.1	14.7 ± 0.0	69.2 ± 0.4	65.8 ± 0.6	64.7 ± 2.5
FPF(emitted) ^b (%)	50.3 ± 0.7	53.0 ± 0.4	18.0 ± 0.2	31.5 ± 0.1	24.9 ± 0.7	22.2 ± 0.9	79.8 ± 0.3	76.6 ± 0.1	73.4 ± 0.9
MMAD ^c (µm)	16.8 ± 0.9	28.4 ± 0.4	14.6 ± 0.1	16.5 ± 0.3	20.0 ± 0.2	11.7 ± 1.3	3.1 ± 0.1	3.6 ± 0.1	4.1 ± 0.3
GSD ^d	4.4 ± 0.2	5.3 ± 0.2	1.8 ± 0.0	3.7 ± 0.0	3.6 ± 0.1	1.8 ± 0.2	3.3 ± 0.0	3.1 ± 0.1	2.4 ± 0.1

^a FPF – fine particle fraction.

^b FPF(emitted) – emitted fine particle fraction.

^c MMAD – mass median aerodynamic diameter.

^d GSD – geometric standard deviation.

The use of AMB in the formulation has benefits in terms of (1) destroying the structure of ASM (Li et al., 2011; Lu et al., 2010), (2) decreasing the bacterial load (via quorum quenching), and (3) allowing effective penetration of the synergistic antibiotics into

the underlying bacteria in mucoid infections. AMB has significant antagonistic quorum-sensing properties (Lu et al., 2010) and in combination with the antibiotics, results in the ability to reduce bacterial cell counts more effectively via enabling drug penetration

Fig. 4. Dose response kill curve of SD-CIP/GAT/AMB/LEU compared to (a) SD-CIP/GAT and (b) SD-AMB/LEU (control) in artificial sputum medium (ASM) spiked with *Pseudomonas aeruginosa*.

and destruction of the biofilm-caged bacteria. As expected, SD-AMB/LEU as a negative control (i.e. non-antibiotic) showed no bactericidal activities (Fig. 4b).

4. Conclusions

A novel ternary antibiotic–(non-antibiotic) DPI formulation was successfully pioneered and optimized for mucoid infections. The spray-dried powder particles were of respirable-size (d_{50} of $\sim 1.61 \pm 0.03 \mu\text{m}$) and slightly corrugated. When dispersed via an Aerolizer® inhaler at 60 L/min, the powder showed concomitant *in vitro* deposition with obtained FPFs of ~ 64 –69%. Favourably, when formulated as a ternary mix, CIP, GAT and AMB showed much higher and uniform FPFs over their singly-delivered counterparts. Tests on ASM spiked with *P. aeruginosa* showed that the optimized ternary formulation (with AMB) was more effective at killing the bacteria (i.e. faster rate of killing) than just the synergistic antibiotics alone (binary formulation; without AMB). The advent of AMB into a synergistic formulation has indeed given the formulator the opportunity to mount a highly beneficial multi-pronged attack on respiratory bacteria, in essence: ‘Penetrate’ (mucociliary clearance), ‘Divide’ (quorum quenching) and ‘Conquer’ (synergistic antibiotics)!

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Pharmaceutics, Drug Delivery and Pharmaceutical Technology

Tailored Antibiotic Combination Powders for Inhaled Rotational Antibiotic Therapy

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ABSTRACT

Respiratory lung infections due to multidrug-resistant (MDR) superbugs are on a global upsurge and have very grim clinical outcomes. Their MDR profile makes therapeutic options extremely limited. Although a highly toxic antibiotic, colistin, is favored today as a “last-line” therapeutic against these hard-to-treat MDR pathogens, it is fast losing its effectiveness. This work therefore seeks to identify and tailor-make useful combination regimens (that are potentially rotatable and synergistic) as attractive alternative strategies to address the rising rates of drug resistance. Three potentially rotatable ternary dry powder inhaler constructs (each involving colistin and 2 other different-classed antibiotics chosen from rifampicin, meropenem, and tigecycline) were identified (with distinct complementary killing mechanisms), coformulated via spray drying, evaluated on their aerosol performance using a Next-Generation Impactor and tested for their efficacies against a number of MDR pathogens. The powder particles were of respirable size (d_{50} , $3.1 \pm 0.3 \mu\text{m}$ – $3.4 \pm 0.1 \mu\text{m}$) and predominantly crumpled in morphology. When dispersed via a model dry powder inhaler (Aerolizer®) at 60 L/min, the powders showed concomitant *in vitro* deposition with fine particle fractions of ~53%–70%. All formulations were successfully tested in the laboratory to be highly effective against the MDR pathogens. In addition, a favorable synergistic interaction was detected across all 3 formulations when tested against MDR *Pseudomonas aeruginosa*.

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Introduction

The increasing occurrence of multidrug-resistant (MDR) bacteria, otherwise known as the “superbugs,” is an alarming public health care threat. Of particular concern are the carbapenem (β -lactam class of antibiotics)-resistant Gram-negative bacteria, which acquire resistance through the enzymatic hydrolysis of carbapenems via carbapenemases.^{1–3} Infections involving these MDR bacteria are often dire in terms of therapeutic options due to the lack of antibiotics that are active against these pathogens. At present, respiratory lung infections due to MDR bacterial pathogens or “superbugs” have very grim clinical outcomes⁴ with mortality rates

as high as 80% for nosocomial and community-acquired pneumonia.^{5,6} Hence, the development of alternative treatment regimens is a pressing need.

Colistin (also known as polymyxin E) is a 50-year-old cationic polypeptide antibiotic that is experiencing clinical revival. During the early years of its clinical use, nephrotoxicity and neurotoxicity⁷ issues diminished its popularity. Its current resurgence as a “last-line” therapeutic against hard-to-treat MDR respiratory pathogens such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Klebsiella pneumoniae*⁸ arises as many of these organisms still retain susceptibility toward it.

However, in recent years, sporadic worldwide reports of colistin resistance have emerged (e.g., the United States,³ Singapore,⁹ Greece,^{10,11} the United Kingdom,¹² and South Korea¹³).³ Therefore, in an effort to extend the therapeutic life of colistin, combination regimens based on colistin as the “anchor” drug as opposed to monotherapy is proposed as a strategy for combating drug resistance. Combinatorial therapy has several benefits. First, from the

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microbiological standpoint, it thwarts the emergence of bacterial drug resistance as the simultaneous development of resistance toward multiple drug classes is remote¹⁴ and second, from a patient management standpoint, it has the potential to reduce colistin toxicity by allowing a lowered therapeutic dose. This is achieved via a synergistic or additive interaction of colistin with its partner antibiotics.^{8,15,16}

When route of administration of antibiotics is considered, inhaled delivery of colistin combinations is more effective in eradicating MDR pathogens as it directly targets the foci of infection. Elevated drug concentrations can be achieved locally at the site of lung infection without the adverse effects of systemic toxicity.¹⁷ A dry powder inhaler (DPI) formulation is superior over the other aerosol delivery modes (e.g., nebulizer or metered dose inhaler) in terms of stability, delivery efficiency, portability, ease-of-use, and the avoidance of undesired precipitation in solutions.^{18–21}

To date, to the best of the authors' knowledge, there has been no commercially available inhaled DPI product for the eradication of respiratory “superbugs.” In addition, no DPI combination involving a synergistic ternary mechanism of action on resistant bacteria (with actual successful testing on resistant species) has ever been reported in the academic literature (previous efforts at best were on binary synergism and tested on nonresistant strains¹⁵). Commercially, the only antibiotic DPI therapies that are currently available or in the pipeline have been limited to just the single species (i.e., tobramycin, colistin, and ciprofloxacin monotherapies),^{15,21–24} and these are clearly not indicated for the specific treatment of MDR pathogens.

In this work, we thereby sought to identify, formulate, and then evaluate the performance of 3 ternary colistin DPI combinations (i.e., colistin/tigecycline/rifampicin [COL/TIG/RIF], colistin/meropenem/rifampicin [COL/MEM/RIF], colistin/meropenem/tigecycline [COL/MEM/TIG]) that could potentially be useful in the fight against respiratory “superbugs.” Colistin was the “anchor” drug, while the other 2 species were chosen from distinct antibiotic classes (conferring distinct killing mechanisms) to make up the “multiple-classed” ternary cocktails (i.e., 3 derived permutations).

Materials and Methods

Materials

Meropenem trihydrate and tigecycline were purchased from Afine Chemical (Hangzhou, Zhejiang, and China). Rifampicin was obtained from Leshan Sanjiu-Longmarch Pharmaceuticals Corporation, Ltd. Colistin sulfate, ammonium acetate, phosphoric acid, and trifluoroacetic were purchased from Sigma Chemical Corporation (St. Louis, MO). Ultrapure water was used in the experiments. HPLC-grade acetonitrile was supplied by Merck (Darmstadt, Germany). Clinical isolates of MDR bacteria used in the study were obtained from the National University Hospital, Singapore, and included *P. aeruginosa* PSE2 (resistant to ciprofloxacin, aminoglycosides, and carbapenems), *K. pneumoniae* ENT443 (resistant to ciprofloxacin and carbapenems), *Escherichia coli* EC424 (resistant to ciprofloxacin and carbapenems), and *Acinetobacter b* AB1 (resistant to ciprofloxacin, aminoglycosides, and carbapenems). Mueller–Hinton broth (Oxoid, Basingstoke, UK) was used as the culture media for the antimicrobial activity test. Etest strips were purchased from bioMérieux SA (Marcy-l'Etoile, France).

Preparation of Spray-Dried Powders

Powders of colistin (SD-COL), meropenem (SD-MEM), rifampicin (SD-RIF), tigecycline (SD-TIG), ternary combination powders of colistin/meropenem/rifampicin (SD-COL/MEM/RIF) in weight

ratio of 5:4:12, colistin/meropenem/tigecycline (SD-COL/MEM/TIG) in weight ratio of 5:4:4, and colistin/tigecycline/rifampicin (SD-COL/TIG/RIF) in weight ratio of 5:4:12 were prepared by spray drying the ethanol–water cosolvent feedstock of antimicrobial agent/s on a B-90 Nano Spray Dryer^{15,25–27} (Büchi Labortechnik AG, Flawil, Switzerland) with operating parameters as detailed in Table 1. The operating parameters for spray drying were adapted from Lee et al.²⁷ A smaller spray mesh size (i.e., 5.5 μm) and more dilute solute concentration (i.e., 0.65 wt/vol %) were applied instead, so as to maximize the production of respirable-size particles (i.e., <5 μm). Higher inlet temperature (i.e., 120°C) was also used to generate drier and hence more aerodynamic powders. Colistin^{28,29} and meropenem^{29,30} are both hydrophilic drugs, whereas rifampicin^{30,31} and tigecycline^{28,32} are lipophilic species. Therefore, appropriate cosolvent choice at a suitable ratio is vital toward the production of uniform ternary combination particles. A water–ethanol mix was selected as the desired cosolvent system in view of the solvents' low toxicity profiles and the drugs' mutual miscibilities in those solvents. The water–ethanol cosolvent volume ratio was adjusted to 2:1 to ensure good solubility of all antibiotics in the cosolvent system.

In this work, *in vitro* susceptibility of control and MDR strains against each individual drug of the different combinations was first determined (Table 2). The minimum inhibitory concentration (MIC) values obtained experimentally were further guided by European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical susceptibility breakpoints³³ (Table 2) to obtain ratios that would be “universally” potent (Table 2). The spray-dried powders were stored in a desiccator at room temperature for further characterization. In this work, colistin sulfate (instead of colistimethate sodium) is chosen as colistin sulfate is more stable than colistimethate sodium in human plasma.³⁴

Surface Morphology

The morphology of the powder particles was examined by a field emission scanning electron microscopy (JEOL JSM-6700; JEOL, Tokyo, Japan) at 5 kV. Before imaging, the samples were dispersed onto carbon sticky tabs and coated with gold for 80 s using a sputter coater (Cressington 208HR, Watford, UK).¹⁵

Particle Size Analysis

The particle size distribution of the spray-dried powders was determined by laser diffraction on the Malvern Mastersizer 2000 (Malvern Instruments, UK) using the Scirocco dry dispersion unit. The powders were dispersed in triplicates at 3 bars of pressure using refractive indices of 1.575 for SD-COL and SD-COL/MEM/TIG, 1.639 for SD-MEM, 1.675 for SD-TIG, and 1.613 for SD-RIF, SD-COL/MEM/RIF, and SD-COL/TIG/RIF.¹⁶ For the combination formulations, the refractive indices were based on the major component in the formulation.

Table 1
Spray-Drying Parameters

Parameter	Value
Spray mesh size (μm)	5.5
Feed concentration (w/v %)	0.65
Cosolvent (DI water:ethanol) ratio (v/v)	2:1
Nitrogen flow rate (L/min)	120
Relative spray rate (mL/h)	4
Inlet temperature (°C)	120
Outlet temperature (°C)	60–65
Yield (%)	80–90

Table 2

MIC Values (Etest) of Colistin, Meropenem, Tigecycline, and Rifampicin Against Various Pathogenic Respiratory Bacteria and Their Reference EUCAST Clinical Breakpoints

Bacterial Species and Strain	MIC Determination (Etest) (mg/L)			
	COL	MEM	TIG	RIF
Control strains				
<i>Pseudomonas aeruginosa</i> ATCC 90207	1	0.125	8	>32 ^a
<i>Klebsiella pneumoniae</i> ATCC 13883	0.125	0.5	0.125	12
<i>Escherichia coli</i> ATCC 25922	0.125	0.032	0.25	16 ^a
<i>Acinetobacter baumannii</i> ATCC 19606	0.25	0.75	0.5	4
Resistant strains				
<i>Pseudomonas aeruginosa</i> PSE2 (CIP ^R GEN ^R MEM ^R)	1.5	3	16 ^a	>32 ^a
<i>Klebsiella pneumoniae</i> ENT443 (CIP ^R MEM ^R) ^b	2	>32 ^a	2	>32 ^a
<i>Escherichia coli</i> EC424 (CIP ^R MEM ^R) ^b	0.5	>32 ^a	1	>32 ^a
<i>Acinetobacter baumannii</i> AB1 (CIP ^R GEN ^R MEM ^R)	1	>32 ^a	2	>32 ^a
Bacterial Species	EUCAST Breakpoint ^c			
	COL	MEM	TIG	RIF
Enterobacteriaceae	>2	>2	>1	NA
<i>Acinetobacter</i> spp.	>2	>2	IE	NA
<i>Pseudomonas</i> spp.	>4	>2	NA	NA
Derived MICs (experimental + EUCAST guided), mg/L	5	4	4	12
		COL/MEM/RIF	COL/MEM/TIG	COL/TIG/RIF
Developed ratios		5:4:12	5:4:4	5:4:12

IE, insufficient evidence that the organism is a good target for therapy with the drug when applied in monotherapy; NA, breakpoints not applicable as the species is considered as a poor target for the drug when applied in monotherapy.

^a This value suggests that the drug when applied in monotherapy is inadequate for targeting the species.

^b Isolate bears a carbapenemase, which results in enzymatic hydrolysis of carbapenems conferring carbapenem resistance.

^c Adapted from literature (http://www.eucast.org/clinical_breakpoints/). Breakpoints indicative of nonsusceptibility.

Powder Crystallinity

Powder crystallinity of the samples was assessed by powder X-ray diffraction at room temperature using an X-ray diffractometer (D8 Advance; Bruker AXS GmbH, Karlsruhe, Germany). Samples were scanned from 5° to 45° (2θ) with an angular increment of 0.04° and at 1 s per step using Cu K_α radiation generated at 35 kV and 40 mA.¹⁶

Surface Area Determinations

The specific surface area of the spray-dried powders was determined by a surface area analyzer (Micromeritics ASAP 2420), using nitrogen as the adsorbate gas. Samples were degassed at 25°C for 24 h on a vacuum dryer, before the measurements. The specific surface area was then determined by the multipoint Brunauer–Emmett–Teller gas adsorption/desorption method.³⁵

Thermogravimetric Analysis

Thermogravimetric analysis studies were carried out using a thermogravimetric analyzer (TGA Q500, TA Instruments). Samples were weighed into a platinum pan and heated in flowing air (90 mL/min) from room temperature to 800°C at a scan rate of 10°C/min. Moisture content (%) was determined from the stable weight loss (%) that occurred at about 100°C.³⁶

Dynamic Vapor Sorption

Moisture sorption isotherms were obtained using the gravimetric vapour sorption (DVS Advantage 1; Surface Measurement Systems, UK) technique. Samples were weighed into a round-bottomed quartz sample pan and exposed to sorption–desorption cycles from 0% to 90% relative humidity (RH) at 25°C and steps of 10% RH. Equilibrium moisture content at each humidity step was determined by a dm/dt of 0.02%/min.³⁷

In Vitro Aerosol Performance

The aerosol performance of the spray-dried powders was assessed using a Next-Generation Impactor (NGI; Copley Scientific, Nottingham, UK) coupled with a United States Pharmacopoeia stainless steel throat in a walk-in environmental chamber (SynerSys, Singapore) maintained at 25°C and 40% RH. The method followed the procedure specified for DPIs in the British Pharmacopoeia.^{15,38} Before testing, all the 8 impactor stages were sprayed with MOLYKOTE[®] 316 silicone grease release spray (Dow Corning Corporation, Midland, MI) to minimize particle bounce. Approximately 10 mg of powder from each formulation was filled into a hydroxypropyl methylcellulose capsule (size 3, Capsugel[®]; Capsugel, Morristown, NJ), loaded into an Aerolizer[®] inhaler (Novartis Pharmaceuticals, Basel, Switzerland), pierced, and then, actuated for 4 s at 60 L/min. Six capsules were fired per experiment, delivering a total dose of 60 mg. The flow through the NGI was measured using a calibrated flow meter (TSI Model 4040C; TSI Instrument Ltd., Buckinghamshire, UK), controlled by a high-capacity vacuum pump (Model HCP5; Copley Scientific) and a critical flow controller (TPK 2000, Copley Scientific). After actuation, the device, capsule, throat, and each part of the NGI were washed separately and thoroughly using ethanol–water cosolvent. The solutions were then assayed by HPLC after appropriate sample dilutions were made. Each dispersion test was performed in triplicates to obtain mean values. At a flow rate of 60 L/min, the aerodynamic cutoff diameters of stages 1, 2, 3, 4, 5, 6, and 7 are 8.06, 4.46, 2.82, 1.66, 0.94, 0.55, and 0.34 μm. Particles with diameter size less than 0.34 μm were captured on a micro-orifice collector beyond stage 7.

In this study, fine particle fraction (FPF) represents the mass fraction of drug particles smaller than 5 μm in the aerosol cloud relative to the total mass recovered and was obtained by interpolation to the cumulative percent undersize at 5 μm. FPF (emitted) was obtained when the fine particle dose was expressed relative to the emitted dose.

Drug Content Quantification

Drug contents in the spray-dried powders and in the aliquots collected from the NGI were analyzed via HPLC (1100 series; Agilent Technologies, CA).¹⁵ Assay of colistin was conducted via a modified HPLC method originally described by Bai et al.³⁹ with Zorbax SB C-18 column ($4.6 \times 150 \text{ mm}^2$, $3.5 \mu\text{m}$; Agilent Technologies) as the stationary phase. The mobile phase consisted of 0.05% (v/v) trifluoroacetic aqueous solution (solvent A) and acetonitrile (solvent B), delivered at 1 mL/min under a linear gradient: 0–8 min, 80%–64% A; 8–10 min, 64% A–80% A. Colistin peak appeared at a retention time of 5.3 min under a UV wavelength of 214 nm. Linearity was obtained between 5 and 80 $\mu\text{g/mL}$ ($R^2 = 0.998$) with a limit of detection (LOD) of 2.60 $\mu\text{g/mL}$ (LOD was calculated based on the SD of the response and slope of the calibration curve [i.e., $\text{LOD} = 3.3 \times \{\text{standard deviation of y-intercept}\}/\{\text{slope of calibration curve}\}$]⁴⁰). A Zorbax Extend C-18 column ($4.6 \times 150 \text{ mm}^2$, $3.5 \mu\text{m}$; Agilent Technologies) was used as the stationary phase for simultaneous assays of meropenem, tigecycline, and rifampicin using a modified method originally described by Bradford et al.⁴¹ The chromatograph separation was performed using a gradient mobile phase system starting with 95:5 solvent A (95% 50-mM ammonium acetate, pH 6.2–5% acetonitrile); B (5% 50-mM ammonium acetate, pH 6.2–95% acetonitrile) changed to 50:50 solvent A:B over 10 min then ramped to 5:95 solvent A:B in 1 min. A 2-min isocratic hold was followed by a step back to the initial conditions. A UV absorbance wavelength of 244 nm was used for the simultaneous detection of meropenem, tigecycline, and rifampicin at retention times of 1.7, 5.7, and 10.6 min, respectively. Linearity was obtained between 5–60 $\mu\text{g/mL}$ ($R^2 = 0.997$), 5–60 $\mu\text{g/mL}$ ($R^2 = 0.999$), and 5–50 $\mu\text{g/mL}$ ($R^2 = 0.999$) with a respective LOD of 2.20, 0.73, and 0.81 $\mu\text{g/mL}$ for meropenem, tigecycline, and rifampicin, respectively. The high mean recoveries (measured concentrations/target concentrations) of about 100% (colistin: 100.19 ± 1.37 ; meropenem: 100.33 ± 1.54 ; tigecycline: 100.31 ± 1.00 ; and rifampicin: 100.39 ± 0.61) for a series of samples with known concentrations and the low relative SDs that are in the range of 0.61%–1.53% confirmed good precision and reproducibility of the assay method.⁴² The lack of additional peaks in the chromatogram suggested the absence of chemical degradation due to the spray-drying process.

Determination of Antimicrobial MIC

The Etest is a plastic strip with a predefined antibiotic gradient immobilized on one side and an MIC interpretive scale printed on the other side. The procedure for MIC determination was carried out as described by the Etest strip manufacturer (bioMérieux). Briefly, bacterial suspensions with a turbidity equivalent to McFarland standard 0.5 were swabbed evenly onto Mueller–Hinton agar plates with a sterile cotton swab. After the surfaces of the plates were dried, the Etest strips of all the tested antimicrobial agents were applied. The plates were incubated at 37°C for 20 h. The MIC value was determined as the value at which there was complete inhibition of growth (i.e., the point where an elliptical zone of growth inhibition intersects the scale). The MIC values were interpreted as susceptible, intermediate, or resistant as according to the EUCAST guidelines for susceptibility testing.

Antimicrobial Activity of Ternary Formulation of Colistin Combinations

In vitro antimicrobial activity of the spray-dried powders was determined quantitatively via time-kill studies. Overnight cultures of the test organism were diluted in Mueller–Hinton broth to give a starting bacterial density of approximately 5×10^5 colony forming

unit/mL.¹⁵ Spray-dried powders were added to the cultures such that the final concentrations were at $1 \times \text{MIC}$ (concentration was chosen to be clinically relevant and achievable *in vivo*; colistin is known to exhibit very active killing of Gram-negative bacteria [even at low doses of $0.5 \times \text{MIC}$],^{43,44} hence the choice to test the drug combinations at $1 \times \text{MIC}$ would seem appropriate). The cultures were then incubated at 37°C with shaking (200 rpm). Bacterial cell counts were estimated at time 0 and 24 h. Synergy was defined as a $\geq 2 \log_{10}$ decrease in colony count after 24 h by the combination compared to the most active single agent, additivity as a $1 < 2 \log_{10}$ decrease in colony count after 24 h by the combination compared to the most active single agent and indifference as a $< 1 \log_{10}$ increase or decrease in colony count after 24 h by the combination compared with that by the most active single agent.^{45,46} All microbiological work was carried out under biosafety level 2 conditions.

Results and Discussions

Identification of Effective Ternary Colistin Combinations

Combination therapy, in particular, synergistic (the combined effect is greater than the sum of the individual effects) and additive (the combined effect is the sum of the individual effects) formulations, is an important innovation in pharmacotherapy,⁴⁷ in the drought of newer antibiotic discoveries to counter the growing threat of antimicrobial resistance. However, identification of suitable candidates at efficacious proportions is crucial and not as straightforward as it seems, as incompatible combinations could lead to antagonistic interactions that might be harmful or even fatal to the patients.⁴⁸

Due to decreased antibiotic development effort within the pharmaceutical industry, colistin is nowadays increasingly and widely recommended as the last-resort therapeutic option against infections caused by MDR Gram-negative pathogens.⁸ In this work, colistin was therefore the anchor drug around (with meropenem, rifampicin, or tigecycline as auxiliaries) which the different colistin combinations (Table 3) were developed and formulated into ternary aerosol dry powders. Meropenem, rifampicin, and tigecycline are among the antibiotics that have clinically proven track records for the treatment of nosocomial infections.^{49–54} Rifampicin is a bactericidal antibiotic drug of the rifamycin group that has been used to prevent and treat tuberculosis and other infections but has very limited use in monotherapy due to the rapid emergence of resistance.⁵⁵ Only in combination with other antimicrobial agents does rifampicin unleash its innate potential in the treatment of serious infections. Meropenem is a member of the carbapenem class of antibiotic that is active against a broad spectrum of Gram-positive and Gram-negative pathogens, including the beta-lactamase producers.⁵⁶ However, the worldwide spread of drug resistance to carbapenem antibiotics^{57–59} is seriously threatening this class of life-saving drugs. Although its use in monotherapy has been undermined, the potential application of this antibiotic in combination with other antibiotics to achieve a favorable interaction (e.g., synergism) is still an untapped valuable resource. Tigecycline, the first in a new class of antibiotics called the glycylcyclines, had been approved by the Food and Drug Administration in 2009 for the treatment of community-acquired pneumonia and was considered a useful alternative against carbapenem-resistant bacteria.⁶⁰ However, its clinical efficacy, especially in severe infections, is a cause of concern and may be due to its subdued bacteriostatic (inhibiting the growth) action (Table 3), low serum levels, and the heightened propensity for resistance development (especially during monotherapy).^{60,61} Although the use of tigecycline in monotherapy does have its

Table 3
Antimicrobial Mechanisms of Action and the Respective Colistin-Anchor Combination Permutations

Individual Drug	Drug Class	Effect on Bacteria	Mode of Action	Ternary Drug Combinations (Drug Ratio by Weight %)
COL	Polymyxin	Bactericidal	Disruption of cell membrane	—
MEM	Carbapenem	Bactericidal	Inhibition of peptidoglycan synthesis	COL/MEM/TIG (5:4:4)
RIF	Rifamycin	Bactericidal	Inhibition of RNA transcription	COL/MEM/RIF (5:4:12)
TIG	Glycylcycline	Bacteriostatic	Inhibition of protein synthesis	COL/TIG/RIF (5:4:12)

limitations, its application in combination therapy is still an unexplored promising opportunity, as the drug could potentially be used to augment the therapeutic properties of the other antibiotic/s in the combination (e.g., as in synergism). Table 2 shows experimentally that meropenem, tigecycline, and rifampicin are clearly not suited for monotherapy against the resistant strains (i.e., heightened MICs). Therefore, applying them to augment the properties of a primary antibiotic (as in combination therapy) is indeed a useful approach.

Table 3 shows the different permutations obtained through mixing the “anchor” drug colistin with the other “adjuvant” antibiotics. Each of the antibiotics in the individual combinations is from a different class and hence would have a different mode of kill on pathogenic bacteria. Figure 1 shows a schematic of the antibiotics’ different mechanisms of action. A combination of antibiotics with a variety of these mechanisms could potentially provide a much broader coverage of kill and prevent the resurgence of superbug resistance. Together with a “toolbox” of 3 ternary combinations that could possibly be rotated by the physician in “rotational therapy,” the possibility of a superbug infection–related relapse would thus be very far remote.

As earlier mentioned, the *in vitro* susceptibility of control and MDR strains against each individual drug of the different combinations was first determined (Table 2). The MIC values obtained experimentally were further guided by EUCAST clinical susceptibility breakpoints³³ (Table 2) to obtain ratios that would be “universally” potent (Table 2). For colistin, the susceptibility breakpoint for *Pseudomonas* spp. was 4 mg/L, and experimentally, the resistant strains were all sensitive to concentrations under 4 mg/L (Table 2). To further enhance the therapeutic efficiency, the derived MIC for colistin was adjusted to 5 mg/L. For meropenem, although the susceptibility breakpoints for Enterobacteriaceae, *Acinetobacter* spp., and *Pseudomonas* spp. were all at 2 mg/L, it was evident experimentally that it was unsuitable to be applied in monotherapy against resistant *K pneumoniae*, *E coli*, and *A baumannii*. As resistant *P aeruginosa* had an MIC of 3 mg/L, the derived MIC for meropenem was therefore adjusted to 4 mg/L to enhance the therapeutic efficiency. For tigecycline, susceptibility breakpoints were not available for *Pseudomonas* spp. and *Acinetobacter* spp. as they were considered fair to poor targets for the drug when applied in monotherapy.³³ Experimentally, tigecycline exhibited a high MIC of 16 mg/L against resistant *P aeruginosa*, thus confirming its monotherapy limitations. Although an MIC of under 3 mg/L was sufficient to eradicate resistant *K pneumoniae*, *E coli*, and *A baumannii*, experimentally, the derived MIC for tigecycline was adjusted to 4 mg/L to enhance the therapeutic efficiency. For rifampicin, there was unavailability of susceptibility breakpoints as the drug was not suited for eradicating Enterobacteriaceae, *Acinetobacter* spp., and *Pseudomonas* spp. when applied in monotherapy.³³ This was confirmed by the experimental data on the control and resistant strains, where high MICs were typically needed for effective killing. The derived MIC for rifampicin was based on the experimental MIC values for *K pneumoniae* ATCC 13883 and *A baumannii* ATCC 19606 (i.e., 4–12 mg/L). The higher value of 12 mg/L was chosen for an enhanced therapeutic efficiency. Consequently, the derived MICs of colistin, meropenem, tigecycline, and rifampicin were 5 mg/L, 4 mg/L, 4 mg/L, and 12 mg/L,

respectively. The drug ratios in the combinations were developed from a ratio of the individual drugs’-derived MIC values (Table 2).

Surface Morphology and Particle Size

The surface morphologies of the single and ternary spray-dried particles are shown in Figure 2. The single spray-dried particles (i.e., SD-COL, SD-TIG, and SD-MEM), with the exception of SD-RIF, were generally spherical in geometry and had corrugated surfaces. Presence of chips was observed for SD-COL (Fig. 2a) and SD-TIG (Fig. 2b), but not for SD-MEM (Fig. 2c). The presence of these chips may be due to particle break-up during spray drying. Interestingly, the morphology of SD-RIF (Fig. 2d) closely resembled a crumpled sheet of paper (Fig. 2d). For the spray-dried ternary combination particles, both SD-COL/TIG/RIF (Fig. 2f) and SD-COL/MEM/RIF (Fig. 2g) had crumpled sheet-like structures, whereas SD-COL/MEM/TIG (Fig. 2e) was a mixture of corrugated spherical particles and irregularly shaped chips.

The similarity in surface morphologies of both SD-COL/TIG/RIF and SD-COL/MEM/RIF to SD-RIF may be due to the dominance of rifampicin in the spray-dried ternary combination particles, which resulted in its prevailing influence on their particle morphologies. Table 4 shows the particle size distribution of the spray-dried formulations. The particle size analysis revealed that all the spray-dried formulations were within the respirable size range, with d_{50} of 2.9 ± 0.1 , 1.6 ± 0.1 , 3.2 ± 0.1 , 2.5 ± 0.1 , 3.1 ± 0.3 , 3.4 ± 0.1 , and 3.1 ± 0.2 μm for SD-COL, SD-MEM, SD-RIF, SD-TIG, SD-COL/MEM/TIG, SD-COL/MEM/RIF, and SD-COL/TIG/RIF, respectively.

Powder Crystallinity

The diffractogram of the raw materials and single and ternary spray-dried powders is shown in Figure 3. The sharp and narrow

Figure 1. Schematic illustrating the antibiotics’ mechanism of action.

Figure 2. Field emission scanning electron microscopy images of spray-dried formulations. (a) SD-COL, (b) SD-TIG, (c) SD-MEM, (d) SD-RIF, (e) SD-COL/MEM/TIG, (f) SD-COL/TIG/RIF, and (g) SD-COL/MEM/RIF.

Table 4Volume Particle Size Distribution (Mean $\pm$ SD, $n = 3$) of the Spray-Dried Formulations

Formulations	d_{10} (μm)	d_{50} (μm)	d_{90} (μm)	Span (μm)
SD-COL	1.1 ± 0.0	2.9 ± 0.1	8.2 ± 0.4	2.5 ± 0.1
SD-MEM	0.9 ± 0.0	1.6 ± 0.1	3.0 ± 0.3	1.3 ± 0.1
SD-RIF	1.2 ± 0.0	3.2 ± 0.1	10.4 ± 1.1	2.8 ± 0.2
SD-TIG	1.1 ± 0.0	2.5 ± 0.1	6.2 ± 0.3	2.0 ± 0.1
SD-COL/MEM/TIG	1.3 ± 0.3	3.1 ± 0.3	9.4 ± 2.1	2.7 ± 1.0
SD-COL/MEM/RIF	1.2 ± 0.0	3.4 ± 0.1	13.5 ± 1.1	3.6 ± 0.4
SD-COL/TIG/RIF	1.4 ± 0.1	3.1 ± 0.2	7.6 ± 0.6	2.0 ± 0.2

Span = $(d_{90} - d_{10}) / d_{50}$. d_{10} , volume diameter under which 10% of the sample resides; d_{50} , volume median diameter; d_{90} , volume diameter under which 90% of the sample resides.

intense diffraction peaks of raw-MEM and raw-RIF indicate their highly crystalline nature, whereas the broad and diffuse peaks of raw-COL and raw-TIG illustrate their amorphous qualities. After spray drying, all the ternary combination powders (i.e., SD-COL/MEM/TIG, SD-COL/MEM/RIF, and SD-COL/TIG/RIF) presented broad diffused diffraction patterns, which were characteristic of amorphous materials. It has been well reported that spray-dried powders are typically produced as amorphous particles.⁶²

Specific Surface Area (Brunauer–Emmett–Teller)

The specific surface areas of the single and ternary spray-dried formulations are shown in Table 5. The specific surface areas of SD-RIF and the ternary spray-dried formulations containing rifampicin (i.e., SD-COL/MEM/RIF and SD-COL/TIG/RIF) were significantly higher than those of the rifampicin-void spray-dried formulations (i.e., SD-COL, SD-MEM, SD-TIG, and SD-COL/MEM/TIG). This could be due to the former's crumpled sheet-like particle structure, which minimized aggregation and promoted high specific surface areas.⁶³ Among the rifampicin-void spray-dried formulations, SD-COL, SD-TIG, and SD-COL/MEM/TIG had slightly

higher specific surface areas (i.e., 10.34 ± 0.67 – 12.97 ± 0.22 m²/g) than SD-MEM (4.35 ± 0.09 m²/g), which could probably be explained by the presence of irregularly shaped chips and hollowed interiors (Fig. 2).

Moisture Content

Table 6 shows the moisture content of the spray-dried powders that were stored at 25 °C/40% RH. SD-RIF and SD-TIG exhibited low moisture contents (i.e., $1.0 \pm 0.0\%$ and $1.0 \pm 0.1\%$, respectively), which could be due to their lipophilicity.^{28,30–32} In contrast, SD-COL and SD-MEM had higher moisture contents (i.e., $8.0 \pm 0.2\%$ and $7.8 \pm 0.3\%$, respectively) as they were hydrophilic drugs.^{28–30,64} Among the ternary combination formulations, SD-COL/MEM/TIG had much higher moisture content (i.e., $6.8 \pm 0.1\%$) as compared to SD-COL/MEM/RIF ($1.6 \pm 0.0\%$) and SD-COL/TIG/RIF ($1.1 \pm 0.2\%$), and this could be due to the higher proportion of hydrophilic drugs (i.e., colistin and meropenem) in the former. The high proportion of lipophilic species (i.e., mainly rifampicin) in the other 2 ternary combination formulations (i.e., SD-COL/MEM/RIF and SD-COL/TIG/RIF) could have contributed to their low moisture contents.

Moisture Sorption Profiles

Moisture sorption isotherms of the single spray-dried formulations (i.e., SD-COL, SD-MEM, SD-RIF, and SD-TIG) and ternary spray-dried formulations (i.e., SD-COL/MEM/TIG, SD-COL/MEM/RIF, and SD-COL/TIG/RIF) are shown in Figures 4a and 4b, respectively. For the single species, both SD-MEM and SD-COL exhibited high maximum mass changes of 37% and 34%, respectively, at 90% RH (Fig. 4a), reflecting the hydrophilicity and high moisture uptake capacity of both materials. SD-MEM and SD-COL were also found to retain some absorbed moisture on desorption completion, which reflected the materials' irreversible water sorption behavior. In contrast, the maximum mass changes of SD-TIG and SD-RIF were

Figure 3. X-ray diffraction patterns of raw-COL, raw-MEM, raw-RIF, raw-TIG, SD-COL, SD-MEM, SD-RIF, SD-TIG, SD-COL/MEM/TIG, SD-COL/MEM/RIF, and SD-COL/TIG/RIF.

Table 5
Specific Surface Areas of the Spray-Dried Formulations

Formulations	Specific Surface Area (m ² /g)
SD-COL	12.97 ± 0.22
SD-MEM	4.35 ± 0.09
SD-RIF	51.27 ± 1.71
SD-TIG	10.34 ± 0.67
SD-COL/MEM/TIG	10.80 ± 1.64
SD-COL/MEM/RIF	36.97 ± 0.41
SD-COL/TIG/RIF	47.48 ± 1.30

understandably lower (i.e., 23% and 9%, respectively), as they were lipophilic species. While the water sorption behavior of SD-TIG was shown to be reversible, the mass loss on desorption exhibited by SD-RIF could have been contributed by the expulsion of residual water.⁶⁵

Among the ternary spray-dried formulations (Fig. 4b), SD-COL/MEM/TIG showed the highest maximum mass change when compared to SD-COL/MEM/RIF and SD-COL/TIG/RIF. As SD-COL/MEM/TIG contained a high proportion (by weight) of colistin in the formulation, it was hence not surprising to note its maximum mass change to be somewhat similar to that of SD-COL (i.e., ~33%). Favorably, SD-COL/MEM/TIG exhibited reversible water sorption behavior, in contrast to SD-COL and SD-MEM. Furthermore, no moisture-induced recrystallization was observed for this formulation. SD-COL/MEM/RIF and SD-COL/TIG/RIF had much lower maximum mass changes (i.e., 19% and 13%, respectively), and their sorption curves were somewhat similar from 0% to 60% RH. For SD-COL/MEM/RIF, the formulation was relatively stable and showed no evidence of moisture-induced recrystallization. The mass loss on desorption could have been due to the expulsion of residual water.⁶⁵ For SD-COL/TIG/RIF, there was a drop in mass after the humidity reached about 60% RH, which indicated recrystallization and the expulsion of absorbed water.^{66–68} Nevertheless, moisture stability for SD-COL/TIG/RIF would not be an issue if it was to be kept under dry conditions (i.e., <60% RH; e.g., in moisture-protective blister packs).

In Vitro Aerosol Performance

The aerosolization performance results of the single and ternary spray-dried formulations are shown in Figure 5 and Table 7. In this work, airflow of 60 L/min was used to simulate the reduced inspiratory effort achieved by patients with compromised lung functions (e.g., pneumonia and cystic fibrosis).^{15,27} Generally, all the single and ternary spray-dried formulations displayed robust delivery efficiency with FPFs ranging from 42.6 ± 1.6% to 69.5 ± 5.0% (Table 7; an FPF value that is significantly higher than 30% is an index of good aerosol performance^{15,69}). In particular, among the single spray-dried formulations, SD-MEM was found to have a slightly lower FPF of 42.6 ± 1.6% when compared to the others (i.e., FPFs > 50%). The lower FPF might be due to the cohesiveness and hygroscopicity of the powder, which could in a way be inferred by

Table 6
Moisture Content of the Spray-Dried Powders That Were Stored at 25°C/40% RH

Formulations	Moisture Content (%)
SD-COL	8.0 ± 0.2
SD-MEM	7.8 ± 0.3
SD-RIF	1.0 ± 0.0
SD-TIG	1.0 ± 0.1
SD-COL/MEM/TIG	6.8 ± 0.1
SD-COL/MEM/RIF	1.6 ± 0.0
SD-COL/TIG/RIF	1.1 ± 0.2

Figure 4. Dynamic vapor sorption isotherm of the (a) single spray-dried formulations and (b) ternary spray-dried formulations.

the formulation's high capsule and device retention (i.e., 12.6 ± 2.8% and 29.0 ± 2.9%, respectively [Fig. 5b])⁷⁰ and sorption characteristics (Fig. 4a). Favorably, when meropenem was formulated as a ternary mix (i.e., SD-COL/MEM/TIG and SD-COL/MEM/RIF), the cohesiveness of meropenem was minimized. Capsule and device retention had decreased significantly to under 5% and 7%, respectively (Figs. 5e and 5f). Clearly, it does seem that formulating the drugs into a ternary mix has the advantage of ameliorating the performance of the poorer-performing species. For the formulations with a crumpled sheet-like morphology (i.e., SD-RIF, SD-COL/MEM/RIF, and SD-COL/TIG/RIF), the dispersibilities obtained were generally robust (i.e., FPFs ranging from 52.8 ± 2.5% to 69.5 ± 5.0%), thus suggesting the favorability of the corrugated and/or crumpled particle morphology in aerodynamics.^{63,71,72} These particles have high free volume with pockets of void space and hence have low densities, are aggregation-resistant, and have reduced interparticle contact areas.^{63,71,72} For the ternary spray-dried formulations (i.e., SD-COL/MEM/TIG, SD-COL/MEM/RIF, and SD-COL/TIG/RIF), there was concomitant deposition observed across all stages (including the throat, inhaler, and capsule), thus suggesting uniformity in the powder and/or aerosol (Figs. 5e–5g). Furthermore, all 3 ternary formulations are highly suited for delivery via the aerosol route in view of the robust FPFs obtained (Table 7). Improvements to the

Figure 5. In vitro deposition of (a) SD-COL, (b) SD-MEM, (c) SD-TIG, (d) SD-RIF, (e) SD-COL/MEM/TIG, (f) SD-COL/MEM/RIF, and (g) SD-COL/TIG/RIF at 60 L/min. Data presented as mean $\pm$ SD ($n = 3$). Stages 1 to 7 denote impactor stages, followed by their corresponding lower aerodynamic cutoff diameter in parentheses. MOC is the NGI micro-orifice collector.

Table 7
Deposition Parameters of Different Formulations Measured by NGI at 60 L/min

Formulations	FPF (%)	FPF (emitted), %
SD-COL	58.5 ± 2.1	69.4 ± 0.6
SD-MEM	42.6 ± 1.6	73.0 ± 1.4
SD-RIF	55.1 ± 3.7	63.6 ± 2.6
SD-TIG	56.0 ± 3.2	70.9 ± 2.2
SD-COL/MEM/TIG		
COL	61.2 ± 4.7	66.8 ± 6.2
MEM	64.9 ± 1.6	70.4 ± 2.2
TIG	62.2 ± 4.4	67.6 ± 5.2
SD-COL/MEM/RIF		
COL	55.9 ± 2.0	60.1 ± 2.3
MEM	52.8 ± 2.5	58.1 ± 2.1
RIF	54.2 ± 2.9	60.3 ± 2.8
SD-COL/TIG/RIF		
COL	69.5 ± 5.0	73.9 ± 6.1
TIG	67.2 ± 5.9	72.5 ± 6.4
RIF	67.8 ± 5.3	72.7 ± 5.9

emitted fraction (i.e., inferred via closely similar FPF and FPF [emitted]) were also achieved in the ternary species, which again reinforces the benefits of codelivery.

Antimicrobial Activity of Spray-Dried Formulations

P aeruginosa is a leading opportunistic pathogen that is mostly responsible for hospital-acquired pneumonia and ventilator-associated pneumonia⁷³ that occurs predominantly in immunocompromised, burn, and cystic fibrosis patients. This pathogen is particularly dangerous and dreaded due to its natural resistance to many antibiotics and its capacity to acquire new resistance mechanisms under pressure from antibiotics exposure.⁷⁴ In recent years, the incidence of resistance to the β -lactams, quinolones, aminoglycosides, and carbapenems had risen steadily worldwide, and at current growth rates, this phenomenon is undoubtedly poised to become a serious global medical threat.^{75–77}

Opportunistic *P aeruginosa* is implicated in many chronic infections and is readily isolated from delayed-healing wounds, medical devices, and the lungs of cystic fibrosis patients. Persistence of *P aeruginosa* is due to its capacity to form biofilms where bacteria embedded in biofilms is protected from antibiotic therapy.^{78,79} Antibiotic combinations had been shown to be more efficient than single antibiotics in treating biofilm-associated infections,^{80–82} and our colistin combinations are predicted to have the same enhanced antibiofilm effects and hence improved antimicrobial activities against biofilm populations of *P aeruginosa*.

In this work, the antimicrobial activities of the ternary spray-dried powders (i.e., SD-COL/MEM/TIG, SD-COL/MEM/RIF, and SD-COL/TIG/RIF) were tested against different MDR pathogenic respiratory bacteria and summarized in Table 8. Overall, the antibiotic combinations showed significant bactericidal effects, in particular, the SD-COL/TIG/RIF combination against *P aeruginosa*, *K pneumoniae*, and *E coli*. Although the combinations' lack of synergistic effects on MDR *A baumannii* could be a strain-specific issue, they are nonetheless, as bactericidally effective as colistin alone.

When all 3 ternary formulations were tested against MDR *P aeruginosa* that was resistant to ciprofloxacin (fluoroquinolone group), gentamicin (aminoglycoside group), and meropenem (carbapenem group), a synergistic kill was obtained across the formulations, with log₁₀ colony forming unit changes of −4.0, −3.6, and −3.2 for SD-COL/TIG/RIF, SD-COL/MEM/TIG, and SD-COL/MEM/RIF, respectively, after a 24-h test period. With a synergistic interaction, a much reduced drug dose would be required to achieve the same therapeutic effect as conventional monotherapy. This is in addition to both the combinatorial and

Table 8
Time-Kill Results of Spray-Dried Formulations Against Different MDR Pathogenic Respiratory Bacteria

Resistant Bacterial Strain (Resistance Profile)	Time-Kill Assay (log ₁₀ change) ^a	Interaction
<i>Pseudomonas aeruginosa</i> PSE2 (CIP ^R GEN ^R MEM ^R)		
SD-COL/TIG/RIF	−4.0	Synergy
SD-COL/MEM/TIG	−3.6	Synergy
SD-COL/MEM/RIF	−3.2	Synergy
<i>Klebsiella pneumoniae</i> ENT443 (CIP ^R MEM ^R) ^b		
SD-COL/TIG/RIF	−1.2	Additivity
SD-COL/MEM/TIG	0.1	Indifference
SD-COL/MEM/RIF	0.0	Indifference
<i>Escherichia coli</i> EC424 (CIP ^R MEM ^R) ^b		
SD-COL/TIG/RIF	−1.0	Additivity
SD-COL/MEM/TIG	0.1	Indifference
SD-COL/MEM/RIF	0.2	Indifference
<i>Acinetobacter baumannii</i> AB1 (CIP ^R GEN ^R MEM ^R)		
SD-COL/TIG/RIF	−0.5	Indifference
SD-COL/MEM/TIG	0.2	Indifference
SD-COL/MEM/RIF	0.2	Indifference

^a Values represent the log₁₀ change in colony forming unit/mL in the time-kill assay after 24-h exposure to the ternary spray-dried formulations compared to the most active drug.

^b Isolate bears a carbapenemase, which results in enzymatic hydrolysis of carbapenems conferring carbapenem resistance.

local delivery strategies (directly to the lungs), which also served to reduce the required drug dose into the body. In totality, a synergistic inhaled combination would not only minimize the toxic side effects of the antibiotic(s) (e.g., colistin) but also help in the minimization of drug resistance.

Although carbapenem-resistant bacteria are currently the bane of patients and medical practitioners worldwide^{1,2} and have limited treatment options, it is nevertheless very encouraging to note that meropenem (i.e., a carbapenem) is still relevant and potent in killing these resistant bacteria when applied in a combinatorial setting. Indeed, this could have positive implications in terms of extending the drug's useful "lifespan." Both SD-COL/MEM/RIF and SD-COL/MEM/TIG (carbapenem [MEM]–containing formulations) were not only bactericidally effective against all 4 species of carbapenem-resistant bacteria (Table 8) but also elucidated a synergistic response for the MDR *P aeruginosa* isolate.

Among the 3 ternary formulations developed in this work, SD-COL/TIG/RIF (noncarbapenem [MEM]–containing combination) was found to be the best performing species. It not only exhibited a synergistic interaction against carbapenem-resistant *P aeruginosa* but also revealed additive interactions against other carbapenem-resistant Gram-negatives such as *K pneumoniae* and *E coli* (Table 8). Although an additive interaction might be a notch lower than a synergistic interaction, they are nonetheless still useful in dosage reduction, which could help in the minimization of the associated side effects and the potential for bacterial resistance. The superior qualities of SD-COL/TIG/RIF (over SD-COL/MEM/RIF and SD-COL/MEM/TIG) may be attributed to colistin, tigecycline, and rifampicin's distinct killing mechanisms, which targeted different functions of the bacterial cell (Table 3 and Fig. 1). Bactericidal colistin is a cationic antibiotic that binds to the anionic lipopolysaccharide of Gram-negative bacteria's membrane, leading to a disruption of the membrane permeability, leakage of intracellular content, and hence, cell death.^{83,84} Perturbation of the bacterial cellular membrane integrity by colistin may in turn favor the uptake of tigecycline and rifampicin to effect a "collaborative" synergistic interaction. Bactericidal rifampicin's mechanism of action is based on its ability to specifically bind and inhibit the bacterial DNA-dependent RNA polymerase.⁴⁹ On the other hand, bacteriostatic tigecycline works by binding to the 30S subunit of the

bacterial ribosome, thus inhibiting peptide elongation and subsequently disrupting protein synthesis.⁵⁰ As each of them (i.e., colistin, rifampicin, and tigecycline) has a unique complementary mode of action that is not shared by any other antibiotics in the combination, it is therefore not surprising to observe a potpourri of favorable interactions (i.e., synergy and additivity) arising from the SD-COL/TIG/RIF species (as compared to SD-COL/MEM/RIF and SD-COL/MEM/TIG; Table 8). For SD-COL/MEM/RIF and SD-COL/MEM/TIG, as they have 2 antibiotics (i.e., meropenem and colistin) with overlapping actions on the bacterial cell wall (Fig. 1), their “collaborative” efficiency is therefore understandably a notch lower than SD-COL/TIG/RIF.

Regular use of antibiotics, be it monotherapy or combination therapy, will eventually lead to resistance. The beauty of the present antibiotic arsenal lies in the potential for “rotational therapy” (i.e., treatment rotated between the 3 different-member combinations). Recalcitrant bacterial infections that require prolonged antibiotic treatment could benefit from such a therapy while enjoying the reduced risk of antimicrobial resistance. With 3 unique combinations involving 3 different complementary antibiotics, the possibility of a bacterium developing resistance to all the antibiotics and combinations simultaneously would hence be very far remote. The present work has focused on and successfully demonstrated the antimicrobial efficacy of the 3 ternary formulations on MDR bacteria. Future investigations will include *in vitro* (mammalian cell lines) studies and *in vivo* animal infection models. The results from these studies will help uncover potential cytotoxicity issues and provide pharmacokinetic insight into the drug combinations, helping to progress the product(s) toward early phase human clinical trials.

Conclusion

A “toolbox” consisting of 3 potentially rotatable ternary DPI combinations (COL/TIG/RIF, COL/MEM/RIF, and COL/MEM/TIG) was successfully tailor-made in the fight against respiratory “superbugs.” The spray-dried powder particles were of respirable size (d_{50} of $3.1 \pm 0.3 \mu\text{m}$ to $3.4 \pm 0.1 \mu\text{m}$) and predominantly corrugated and/or crumpled in morphology. When dispersed via an Aerolizer® inhaler at 60 L/min, the powders showed concomitant *in vitro* deposition with obtained FPFs of ~53%–70%. In this work, the benefits of codelivery include the potential for synergy, which helps to further lower the toxic drug dose and the associated side effects (i.e., colistin), ameliorating the performance of a poorer-performing species (e.g., cohesiveness and dispersibility) (i.e., meropenem), the life-span extension of an “expiring and/or redundant” antibiotic (i.e., meropenem), and the opportunity to mount a collaborative and concerted mode of attack on bacteria (i.e., COL/TIG/RIF). The advent of these novel formulations is indeed timely and will no doubt equip the human race with the necessary tools to confront the “plague of the 21st century” with renewed confidence.

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