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RESEARCH PAPER

# A Rifapentine-Containing Inhaled Triple Antibiotic Formulation for Rapid Treatment of Tubercular Infection

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## ABSTRACT

***Purpose*** The potential for rifapentine-containing oral therapeutic regimens to significantly shorten the current six-month anti-tubercular treatment regimen is confounded by high plasma protein binding of rifapentine. Inhaled aerosol delivery of rifapentine, a more potent anti-tubercular antibiotic drug, in combination with other first-line antibiotics may overcome this limitation to deliver a high drug dose at the pulmonary site of infection.

***Methods*** A formulation consisting of rifapentine, moxifloxacin and pyrazinamide, with and without leucine, was prepared by spray-drying. This formulation was assessed for its physico-chemical properties, *in vitro* aerosol performance and antimicrobial activity.

***Results*** The antibiotic powders, with and without leucine, had similar median aerodynamic diameters of 2.58 ± 0.08 μm and 2.51 ± 0.06 μm, with a relatively high fine particle fraction of 55.5 ± 1.9% and 63.6 ± 2.0%, respectively. Although the powders were mostly amorphous, some crystalline peaks associated with the δ polymorph for the spray-dried crystalline pyrazinamide were identified.

***Conclusions*** Stabilisation of the powder with 10% w/w leucine and protection from moisture ingress was found to be necessary to prevent overt crystallisation of pyrazinamide after long-term storage. *In vitro* biological assays indicated antimicrobial activity was retained after spray-drying. Murine pharmacokinetic studies are currently underway.

**KEY WORDS** inhaled aerosol · rifapentine · treatment shortening · tuberculosis

## INTRODUCTION

The World Health Organisation treatment regimen for active tuberculosis (TB) comprises of a series of first-line oral antibiotics, consisting of rifampicin, isoniazid and pyrazinamide in high doses (for a 60 kg adult, 600 mg, 300 mg and 1,500 mg, respectively), administered daily for 6 months (1). Although highly effective when administered correctly, the lengthy treatment time along with side effects contributes to early self-termination of treatment by patients and the risk of developing drug-resistant tuberculosis (2,3). Currently there are 1.4 million deaths from TB per annum worldwide. The future of successful treatment of TB therefore involves considerable shortening of treatment duration and minimizing drug side effects. Recent studies have demonstrated that such a drastic change may be possible by replacing two of the standard drugs used in the first-line regimen, rifampicin and isoniazid, with rifapentine and moxifloxacin, respectively (2,4–7).

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## Rifapentine

Rifampicin is recognised as one of the most important first-line sterilising agents for TB. Its relatively short half-life (2–4 h), however, necessitates frequent dosing (2). Cyclopentyl substitution of a methyl group of rifampicin produces rifapentine, a rifamycin with a substantially extended drug half-life (13–14 h) and improved antimicrobial activity so that it is currently administered orally once-weekly (2,8).

Interestingly, when rifapentine was administered daily, the more frequent dosing exhibited potential for more rapid resolution of tubercular infection. Rosenthal *et al*. (2007, 2008) (2,6) used a murine model of tubercular infection to demonstrate that oral rifapentine, in conjunction with pyrazinamide and either isoniazid or moxifloxacin, was four times more active than rifampicin. The rifapentine-containing formulations facilitated successful treatment of BALB/c mice with active tubercular infection in just 3 months or less, when given daily in lieu of rifampicin (2,6). This is important as treatment times with current anti-tubercular drug regimens are derived from *Mycobacterium tuberculosis* (*M.tuberculosis*)-infected murine models (9).

Surprisingly these shortened times were not replicated in more recent animal studies and human clinical trials. An ongoing human clinical trial (Tuberculosis Trials Consortium Study 29) showed rifapentine and rifampicin were not significantly different at equivalent dosing, when used in combination with isoniazid, pyrazinamide and ethambutol (10). Using a guinea pig model of active *M.tuberculosis* infection, Dutta *et al*. (2012) similarly was unable to replicate the treatment shortening properties of rifapentine (11). These discrepancies may partly be explained by the pattern of lung pathologies in infected humans and guinea pigs, where active infection includes extracellular infection within necrotic lung granulomas (12,13). Contrastingly, necrotic granulomas or cavities are absent in BALB/c mice where the *M. tuberculosis* infection is primarily intracellular (11). Because rifapentine accumulates significantly intracellularly compared to rifampicin, its activity may have favoured it against murine infection.

However, a follow-up study in granuloma-forming C3HeB/FeJ mice, still produced short treatment times with rifapentine, comparable to the aforementioned study in BALB/c mice (14). This unexpected result was attributed to the relatively small size of murine granulomas. Rifapentine is 97–98% protein bound in plasma, leaving approximately ten times less free drug than rifampicin (15–17). As only unbound rifapentine can diffuse into necrotic lesions, this effectively constitutes a lower than expected bactericidal activity at the equivalent dose of rifampicin (18). Moreover, the protein binding effect is more likely to be problematic in the substantial tubercular granulomas found in the lungs of the guinea pig model and human patients. Indeed, even though rifapentine is four times more potent than rifampicin in the murine model, in humans the dose-efficacy profile of the two drugs was similar (10,19). When rifapentine is given orally, the combination of high plasma protein binding along with extracellular tubercular infection in necrotic lesions means that shortening of human tubercular treatment is unlikely. Recent studies are investigating whether these obstacles might be overcome by increasing the dose of rifapentine (14,18,20).

## Moxifloxacin

Moxifloxacin is another antitubercular antibiotic favoured for its pharmacokinetic profile (serum half-life 10–12 hrs) and limited drug-drug interactions (9). When, in the standard treatment regimen, moxifloxacin replaced ethambutol with rifampicin, isoniazid and pyrazinamide, it led to improvements in early stage sputum culture conversion and enhanced regimen sterilising activity (21–23). These studies suggest moxifloxacin has treatment shortening potential. In addition, a cure (defined as lack of lung colony forming units at 6 months after treatment) was achieved with moxifloxacin after 4 months, compared to 6 months for isoniazid, when administered during the intensive phase of therapy in a murine model of *M. tuberculosis* infection (4,5). However, a human clinical trial substituting moxifloxacin for isoniazid in the same standard treatment with rifampicin and pyrazinamide, was unable to demonstrate a significant difference between the two drugs in the rate culture negativity at 8 weeks (9). Nevertheless, various *in vitro* and *in vivo* (human and murine) studies suggest moxifloxacin has efficacy at least comparable to isoniazid (2,6,24–27) and may also possess sterilising activity not found with isoniazid (28). This equivalency is significant as isoniazid is one of the key drugs in current combination therapy.

Importantly, moxifloxacin is pharmacokinetically compatible with rifapentine and has demonstrated a similar safety profile to isoniazid in human trials (9,22). When combined with a rifamycin, moxifloxacin has shown some antagonistic activity against cell kill rate, but this is offset by synergism in suppressing development of drug-resistant strains of *M. tuberculosis* (29,30). This is not unlike the partial antagonistic action of isoniazid against the bactericidal activities of rifampicin and pyrazinamide (9,31,32). When co-administered with once or twice-weekly rifapentine in patients with pulmonary tuberculosis, moxifloxacin experienced slightly increased systemic clearance. However, no clinically significant change to overall moxifloxacin exposure was detected, which is important as the area under the plasma concentration curve (AUC) for moxifloxacin is the primary driver of activity (9,33). Of the four clinical trials evaluated by Fouad *et al*. (2011), moxifloxacin was well tolerated by patients (26). For treatment of multi-drug resistant tuberculosis (MDR-TB), moxifloxacin may be the

only drug in the regimen with efficacy and safety that matches that of first-line drugs (34). With a safety profile similar to isoniazid, compatible pharmacokinetics with rifapentine and potential treatment shortening potential, moxifloxacin is a strong replacement candidate for isoniazid when used in a formulation with a rifapentine and pyrazinamide (2,6).

### Pyrazinamide

Pyrazinamide is a key first line anti-tubercular drug with significant sterilising activity used in the intensive phase of active tuberculosis treatment (35). Its use with rifamycins is well-established, whereby synergistic efficacy when administered with rifampicin is responsible for shortening tuberculosis treatment, from 9 months to the current standard 6 month regimen (36). In particular, it plays a crucial role as the only anti-tuberculosis drug able to kill dormant tuberculosis bacilli more effectively than those that are actively metabolizing (37). Similar to the rifamycins, recent studies suggest increasing the current dose of pyrazinamide may further improve efficacy (36,38,39). Until recently however, oral doses were considered at the maximum owing to the association of pyrazinamide with increased hepatotoxic events at high doses (39). With an unclear association between adverse events and oral doses, inhalable forms of pyrazinamide have been suggested as an alternative way to boost pulmonary pyrazinamide concentrations without drastically increasing drug load (37,40).

### Inhaled Anti-Tubercular Therapy

Administering a rifapentine-containing formulation by inhalation may amplify drug exposure at the pulmonary site of infection, whilst minimising the drug burden and bypassing problematic systemic drug-protein binding. In addition, aerosolised medication is logical for patients with TB since 75–80% have infection localised in the lungs (41–43). It may allow faster resolution of the disease whilst reducing related drug load and related side effects (44–46). To this end, anti-tubercular drug delivery by inhalation has focused on two approaches tested both *in vitro* and *in vivo* animal infection models: 1) Inhalable particles co-formulated with drug-release controlling polymers, such as poly(D, L-lactide-co-glycolide) (PLGA) for slow-release of anti-tubercular drugs (47–52); and 2) Microparticles with high drug loading (>75% drug) to maximise drug dosing to the lung (45,46,53–55). Our work focuses primarily on the latter approach.

Previously, it was demonstrated that three first-line anti-tubercular drugs - rifampicin, isoniazid and pyrazinamide - could be formulated into a single inhalable formulation (56). However, this formulation lacked long-term stability owing to the degradation of rifampicin and isoniazid. When formulated together, the drugs react to form an isonicotinyl hydrazone (RIH) adduct in a process that is significantly catalysed by the presence of the first-line antibiotics pyrazinamide and ethambutol (44,57,58).

Our current formulation makes significant improvements on the previous one. Firstly, rifampicin is replaced with the more efficacious rifapentine, possibly shortening treatment time. Additionally, the greater relative *in vivo* potency of rifapentine may potentially reduce inhaled drug load. Secondly, since a similar detrimental chemical reaction with isoniazid is expected to occur with rifapentine (57), isoniazid was replaced with moxifloxacin, given that rifapentine was found to be similarly efficacious when used in combination with either isoniazid or moxifloxacin (6). Finally, we investigated the effects on aerosol performance and storage stability when leucine was incorporated into the formulation. In the present study, a novel, improved and chemically stable inhaled triple antibiotic containing rifapentine, moxifloxacin and pyrainzamide, with potential to significantly reduce treatment duration for TB, is presented.

## MATERIALS AND METHODS

### Dry Powder Manufacture

The antibiotic dry powder was produced by spray-drying. The distinct solubilities of the three drugs required selection of an appropriate solvent. An ethanol:water (50:50) co-solvent was found suitable, whilst being relatively non-toxic. Drug solubilities in this co-solvent were found to be 3.3, 18 and 12 mg/mL at 25°C for rifapentine, moxifloxacin and pyrazinamide, respectively (data not shown). The feed solution consisted of rifapentine (3 mg/mL), moxifloxacin hydrochloride (2 mg/mL) and pyrazinamide (7.5 mg/mL) (Hangzhou ICH Imp & Exp Co. Ltd., Hangzhou, China) solubilised in an ethanol:water (50:50 v/v) mixture. For the powder containing 10% w/w leucine, 1.4 mg/mL of L-leucine (Fluka, Sigma-Aldrich, Castle Hill, Australia) was additionally solubilised. The three antibiotics and leucine were also individually spray dried for thermal analysis. Spray-drying was undertaken with a Buchi-290 Mini spray-dryer coupled in series with a Buchi-296 dehumidifier and Buchi B-295 inert-loop (all from Buchi Laboratories, Flawil, Switzerland), using nitrogen as the drying gas. The following spray-dryer settings were used: inlet temperature 60°C, atomiser 45 mm (approximately 540 L/h), aspirator 100% (40 $m^3$/h) and feed rate 4% (1.4 mL/min). Powders were protected from light and moisture in an opaque desiccator container at 25°C and used within 3 days of manufacture.

### Scanning Electron Microscopy

Particle surface morphology of the samples were characterised using a Zeiss Ultra Plus scanning electron microscope (Carl Zeiss, Oberkochen, Germany) operated at an acceleration voltage of 10 kV. Powders were prepared for imaging by dispersion onto carbon tape which was sputter-coated with approximately 15 nm of gold (Emitech K550X).

### Particle Sizing

The volumetric median diameter (VMD) and span (defined as the difference between the 10th and 90th percentile particle diameters) of the sample powders were analysed by laser diffraction (Malvern Mastersizer 2000, Malvern Instruments Ltd., UK). Powders were dispersed for sizing via the Scirocco 2000 dry powder accessory. A dispersive air pressure of 3.5 bar was chosen as optimal after assessing median particle size over a range of pressures (0.5–4.0 bar).

The refractive index (RI) for pyrazinamide (1.5) was chosen for measurement, as the drug constitutes the majority of the antibiotic powder by weight and varies little from the RIs of rifapentine (1.6) and moxifloxacin (1.6) (40). Measurements were performed in triplicate.

### Aerosol Dispersion

The methods for aerosol dispersion using a multi-stage liquid impinger (MSLI) (Copley Scientific, Nottingham, UK) and quantification of the sample powders, were adapted from Chan *et al*. (2012) (56).

Briefly, the first four MSLI stages of the impinger apparatus were each filled with 20 mL of rinsing solvent (methanol:50 mM phosphate buffer (pH 3.0) (50:50 v/v)), whilst the fifth stage was fitted with a 0.2 μm glass filter (Pall Corporation, Australia). An Aeroliser® dry power inhalation (DPI) device (Novartis, Australia) was connected to the USP throat of the MSLI by a mouthpiece adapter. Twenty milligrams of sample powder, filled in a size 3 hydroxypropyl methylcellulose capsule (Capsugel, USA), was actuated through the DPI device for 2.4 s at an airflow of 100 L/min calibrated using a pump and flow meter (GAST Rotary vein pump, Erweka GmbH, Germany; TSI 3063 airflow meter, TSI instruments Ltd, Buckinghamshire, UK). The airflow of 100 L/min was chosen instead of the standard 60 L/min to maximise aerosolization of the powder. Furthermore, it has been proven that an airflow of 100 L/min is readily achievable by patients using this device (59). The device, capsule, throat, and stages 1–5 of the MSLI were washed using specific amounts of the rinsing solvent. Each sample was tested in triplicate.

A Shimadzu high performance liquid chromatography (HPLC) system comprised of a CBM-20A controller, LC-20AT pump, SPD-20A UV/VIS detector, SIL-20A HT autosampler and LCSolution software (Shimadzu Corporation, Kyoto, Japan) was used to quantify the aerosol drug deposition. This was coupled with a μBondapak™ C18 (3.9×300 mm) column (Waters, Milford, MA, USA) with a sample injection volume of 20 μL.

A gradient method was adapted from Chan *et al*. (2012) (56) using the following mobile phases: (A) 50 mM phosphate buffer (pH 3.0) (orthophosphoric acid from Ajax Finechem Pty Ltd, Taren Point, Australia; potassium dihydrogen orthophosphate from Biolab Ltd, Clayton, Australia), (B) acetonitrile and (C) methanol (V.S. Chem House, Bangkok, Thailand). The gradient profile was A:C (1:1 v/v) for 10 min, followed by a linear decrease to A:C (1:0 v/v) in conjunction with an increase to A:B (3:7 v/v) by 13 min. The latter was maintained for 7 min followed by a gradient to A:C (1:1 v/v) by 23 min which was maintained until 30 min. The flow rate was operated at a constant 1.0 $mL \cdot min^{-1}$.

The UV wavelengths for detection were adjusted as follows: 265 nm for pyrazinamide at 3.5 min, then 216 nm from 5 min followed by 256 nm at 10 mins, to detect moxifloxacin and rifapentine at 6.2 min and 16.7 min, respectively. Standards solutions between 0.001 to 0.4 mg/mL were prepared for each of the three antibiotics, and all gave an $R^2$ value of 1.00.

Aerosol performance properties including the fine particle fraction (FPF) and mass median aerodynamic diameter (MMAD) were calculated using the total emitted dose which is defined as the post-aerosolisation weight difference between the initial loaded drug weight and the amount of drug retained within the Aeroliser® and capsule. The MMAD was obtained from the log-probability plot of the MSLI results. FPF describes as the percent mass of aerosol particles with an aerodynamic diameter less than 5 μm.

### X-ray Powder Diffractometry

Powder crystallinity was determined by X-ray powder diffractometry (XRPD) (D5000, Siemens, Karlsruhe, Germany). The sample powder was distributed into the cavity of a XRPD glass slide for analysis from 5 to 40° $2\theta$, using Cu Kα radiation (30 mA, 40 kV) at a step rate of 0.04° $2\theta$ per second.

### Thermal Analysis

Thermal response profiles for the sample powder were assessed by differential scanning calorimetry (DSC) (821e; Mettler Toledo, Melbourne, Australia). Five milligrams of sample powder was loaded into an aluminium crucible and crimped with a perforated lid. The crucible was heated from 40 to 400°C at 10°C /min. STARe software V.9.0x (Mettler Toledo, Greifensee, Switzerland) recorded related thermal

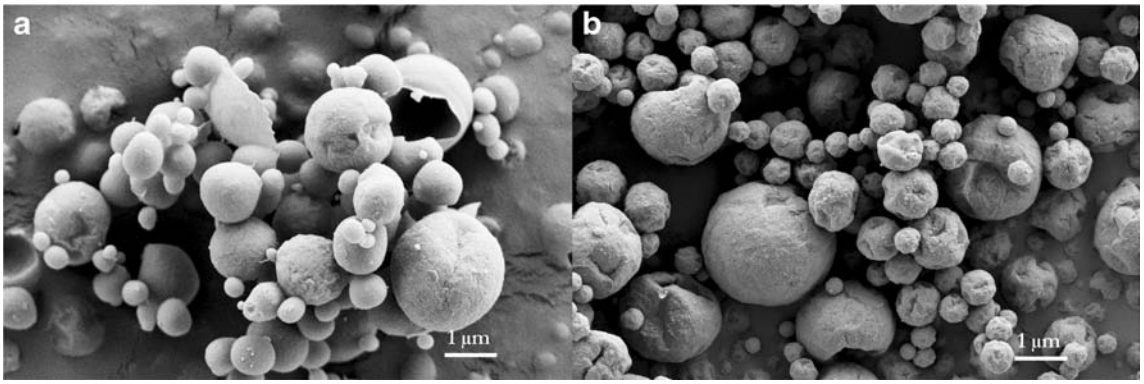


**Fig. 1** Scanning electron micrographs of (**a**) RMP (**b**) RMPL.

data including the exothermal and endothermic peak temperatures.

### Dynamic Vapour Sorption

A Dynamic Vapour Sorption (DVS) instrument (Surface Management Systems, London, United Kingdom) was used to derive the moisture sorption isotherms of the sample powders. The chamber was maintained at 25°C under continuous nitrogen flow. Ten milligrams of each sample was dried at 0% RH for 1 h before being exposed to dual cycles of 0–90% RH, with 10% RH increments. Equilibrium moisture content was determined as a dm/dt of 0.002% per minute. Mass changes were recorded over time.

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### Resazurin Assay

Susceptibility testing of *M. tuberculosis* to the spray dried antibiotic formulations was assessed by resazurin assay. Prior to use in resazurin reduction assays (60), *M. tuberculosis* H37Ra (ATCC 25177) was cultured at 37°C to mid-exponential phase (OD600 0.4–0.8) in Middlebrook 7H9 (Difco Laboratories, Detroit, MI, USA) broth medium, supplemented with albumin-dextrose-catalase (ADC; 10% v/v), 0.05% glycerol, and 0.05% Tween-80. The culture was diluted to OD600 0.002 in 7H9S media (Middlebrook 7H9 with ADC, 0.05% glycerol, 0.05% Tween-80, 1% tryptone) and $2\times10^4$ CFU/ml added to wells of a 96-well microtitre plate. The drug formulations were added after dissolving in 100% DMSO and serially diluting to appropriate concentrations in 7H9S. After incubation for 5 days at 37°C in a humidified incubator, 0.02% resazurin solution (30 µL) and 20% Tween-80 (12.5 µL) was added to each well. Sample fluorescence was measured after 48 h on a BMG Labtech Polarstar Omega instrument with excitation at 530 nm and emission at 590 nm. After subtracting background fluorescence, percentage growth was determined in comparison to growth of uninhibited H37Ra.

The minimum inhibitory concentration (MIC) was defined as the lowest drug concentration necessary to completely inhibit detectable growth of H37Ra.

### Powder Stability Testing

The prepared powders were stored at 0% or 60% relative humidity (RH) in loosely capped glass scintillation vials at 25°C to allow air and moisture exchange between vial and container. Specifically, for 0% RH, the sample was kept under vacuum in a desiccator with silica gel desiccant beads, whilst powders at 60% RH were stored in a climate controlled cabinet. In both cases, samples were protected from light. Aerosol

**Table I** Aerosol Powder Properties of RMP and RMPL Derived from Laser Diffraction and MSLI Dispersion

| | Laser Diffraction | | MSLI | | |
|---|---|---|---|---|---|
| Formulation | VMD (µm) | Span | MMAD (µm) | GSD | FPF (%) |
| RMP | 1.79 ± 0.05 | 2.30 ± 0.36 | 2.58 ± 0.08 | 2.03 ± 0.05 | 55.5 ± 1.8 |
| RMPL | 1.82 ± 0.03 | 1.95 ± 0.11 | 2.51 ± 0.06 | 1.94 ± 0.05 | 63.6 ± 2.0 |

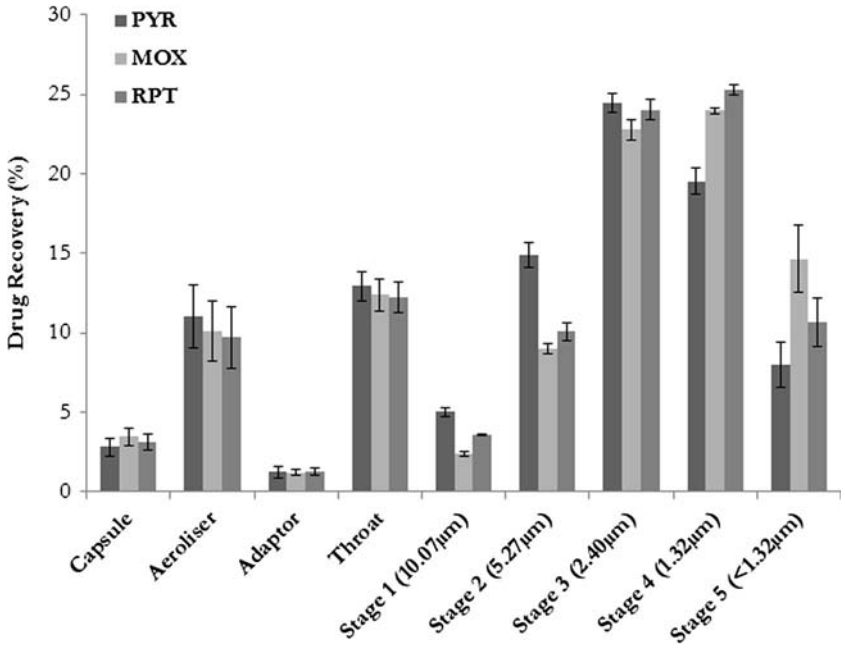


**Fig. 2** Aerosol deposition profile of RMP at 0 months, following MSLI dispersion at a flow rate of 100 L/min ($n = 3$). For each stage, levels of individual antibiotics are shown as a percentage of its own total actual recovered amount. Error bars show standard deviation.

dispersion properties and drug content were assessed up to a 3-month time point according to the aforementioned methods.

## RESULTS

### Antibiotic Powder Properties

The triple antibiotic powders produced, with (RMPL) and without leucine (RMP), are suitable for aerosolisation. Their morphologies are presented in Fig. 1a and b, respectively. Physical particle sizes of both powders were similar, ranging from approximately 200 nm to 3 μm which is suitable for targeting infection down to the deep alveolar regions of the lung. This corresponds to a volumetric median diameter (VMD) of 1.79±0.05 μm and 1.82±0.03 μm, and a span of 2.30±0.36 and 1.95±0.11, for RMP and RMPL, respectively (Table I). Importantly, both sets of particles are spherical and particles are distinct, suggesting a lack of particle agglomeration. Particles containing drug-only (Fig. 1a) had a

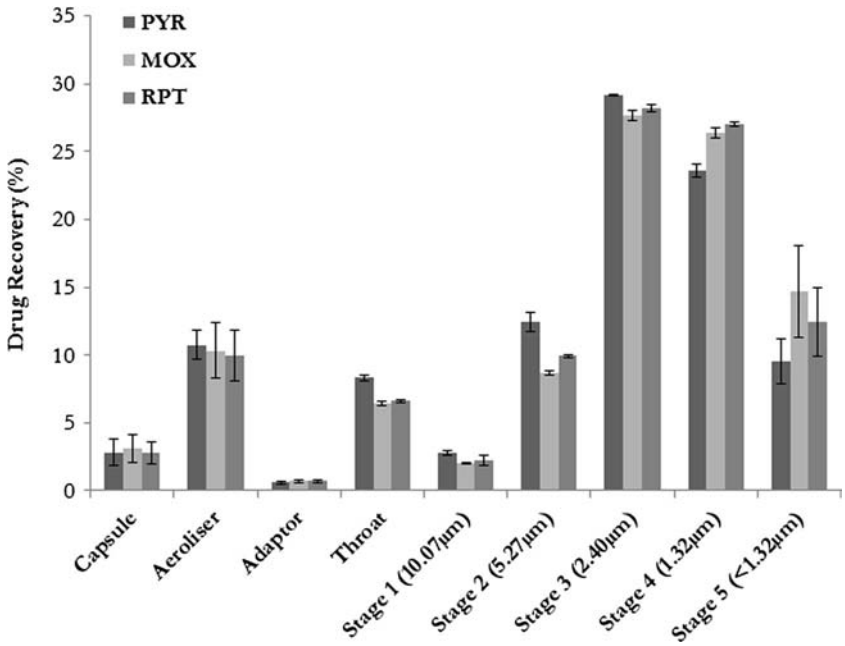


**Fig. 3** Aerosol deposition profile of RMPL at 0 months, following MSLI dispersion at a flow rate of 100 L/min ($n = 3$). For each stage, levels of individual antibiotics are shown as a percentage of its own total actual recovered amount. Error bars show standard deviation.

**Table II** Minimum Inhibitory Concentrations (MICs) of the Individual Antibiotics, Controls and Spray Dried Formulations

| Drug/Formulation | MIC (ng/mL) |
|---|---|
| RMP | 4 |
| RMPL | 4 |
| Rifampicin | 1 |
| Rifapentine | 1 |
| Moxifloxacin | 63 |
| Pyrazinamide | N/A (up to 400 μg/mL) |
| Leucine | N/A (up to 35 ng/mL) |

smooth surface, whilst the introduction of leucine resulted in a corrugated particle surface (Fig. 1b). The enhanced aerosolisation properties of RMPL are observed in its fine particle fraction (Table I), which is 8.1% higher than for RMP without leucine. The FPF was 55.5±1.8 and 63.6±2.0% for RMP and RMPL, respectively.

Interestingly, the MMAD and geometric standard deviation (GSD) of 2.58±0.08 μm and 2.03±0.05 for RMP, and 2.51±0.06 μm and 1.94±0.05 for RMPL, were similar. Comparison of the aerosol deposition profiles (Figs. 2 & 3) for the two powders, show this is primarily due to a 5.4% reduction in throat deposition.

### *In Vitro Antimicrobial Activity*

The MICs of the individual drugs, leucine, rifampicin control and the two spray-dried formulations, are outlined in Table II. The MIC is strain-dependent and not directly related to *in vivo* activity (61). Rifapentine and the rifampicin control had an MIC of 1 ng/mL, whilst moxifloxacin inhibited growth at 63 ng/mL. Notably, antimicrobial activity of pyrazinamide was not detectable even at concentrations up to 400 μg/mL. Leucine did not have a detectable effect on bacterial growth even at up to 35 ng/mL, nor did it appear to inhibit the activity of the other antibiotics.

### *Powder Physico-Chemical Stability Study*

Stability studies (Table III) indicated statistically significant ($p < 0.05$) changes in MMAD and GSD for RMP stored at 60% RH, which contribute to a drastic 10% reduction in FPF. In comparison, these initial values were retained for RMP at 0% RH and RMPL at 0 and 60% RH conditions. However, these values are non-specific and do not reflect significant changes observed in the aerosol deposition profiles for RMP and RMPL stored at 60% RH (Figs. 4, 5 and 6). In these profiles, individual antibiotic concentrations of the different MSLI stages were found to have shifted considerably. Specifically, there is a clear shift of pyrazinamide from the smaller, respirable-sized drug particles (Stages 4 and 5; MMAD < 2.4 μm, Fig. 5) into the larger size fractions (Throat and, Stages 1 and 2; MMAD > 5.72 μm, Fig. 6). Figure 7, an SEM image of RMP stored at 60% RH for 3 months at 25°C, shows bulky pyrazinamide crystals, which were initially absent after spray-drying, are observed to have formed out of the original spherical particles at 3 months.

These observations correlate with the XRPD data for RMP and RMPL. Both powders impart a similar, mostly amorphous 'halo' pattern, combined with several small intensity peaks (in particular 2θ = 16.3 and 27.7°), which correlate with the δ polymorph for spray-dried crystalline pyrazinamide (Fig. 8) (40,56,62). However, when XRPD was performed again following storage at 0 and 60% RH for 3 months, increased peak intensities were recorded at 9.1, 16.4, 18.3, 20.0, 22.4, 23.5, 27.7° in the x-ray diffractograms for the two powders. These changes were much more pronounced for the powder stored at 60% RH.

In comparison, the aerosol deposition profiles for the powders stored at 0% RH (Figs. 9 and 10) did not appear to change significantly from their initial MSLI powder deposition after aerosolisation. However, detailed analysis of individual drug concentrations in the MSLI components containing the respirable fraction of particles (FPF, Stage 3 to 5, containing particles < 5 μm) (Figs. 9 and 10; Table IV) indicates that a noticeable change in drug ratios occurred, although to a markedly lower amount. The leucine-containing formulation at 0% RH did not show similar change even after 3 months storage.

Moisture sorption profiles for RMP and RMPL (Figs. 11 and 12) show the two powders experience a maximum mass change of 6.8% and 6.4%, respectively, at 90% RH. Similar reversible moisture sorption was observed for both formulations, with slight variations between the hysteresis of the first and second moisture sorption profiles.

**Table III** Aerosol Powder Properties of RMP and RMPL After Storage at 0% and 60% RH for Three Months

| | 0% RH | | | 60% RH | | |
|---|---|---|---|---|---|---|
| Formulation | MMAD (μm) | GSD | FPF (%) | MMAD (μm) | GSD | FPF (%) |
| RMP | 2.63±0.11 | 1.98±0.01 | 58.4±1.3 | 3.17±0.12 | 2.55±0.19 | 43.5±1.6 |
| RMPL | 2.62±0.03 | 2.00±0.00 | 63.3±1.2 | 2.47±0.19 | 1.96±0.14 | 61.4±2.7 |

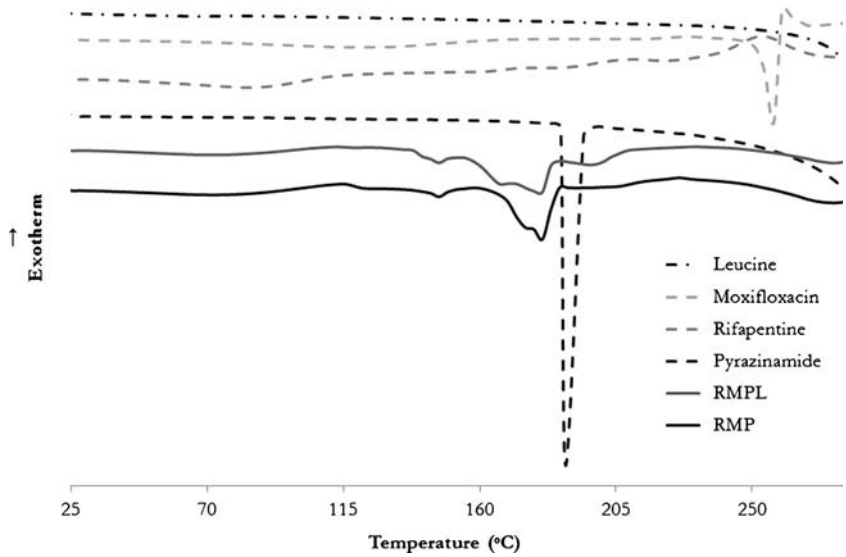


**Fig. 4** DSC thermograms for RMP and RMPL directly after spray drying and individual spray-dried components.

Thermal properties of RMP and RMPL are compared to the individual spray dried components in Fig. 4. Both spray dried moxifloxacin and rifapentine exhibit broad endotherms at 45 – 172 and 40 – 140°C. Rifapentine undergoes a baseline shift at around 161–173°C whilst moxifloxacin and pyrazinamide experience sharp endothermic peaks at 188 and 257°C, respectively. Spray dried leucine shows an extended endotherm from 25 to 280°C. RMP and RMPL exhibit multiple endothermic peaks between 112 and 188°C. Glass transition temperatures were recorded for spray dried moxifloxacin and rifapentine at 178 and 166°C, respectively. Two small endothermic peaks were observed at 140 and 151°, which were consistent with previously reported results of solid-solid phase transitions to the α and γ polymorphs, respectively (40).

## DISCUSSION

A single inhaled formulation containing multiple drugs can offer benefits, but be complex to formulate. In the current study, the three antibiotics have proven synergistic efficacy *in vivo* against TB, with leucine added to enhance physical stability and aerosol performance (63). However, the varying properties of these compounds, including different solubilities, made it challenging to spray-dry. For example, the low

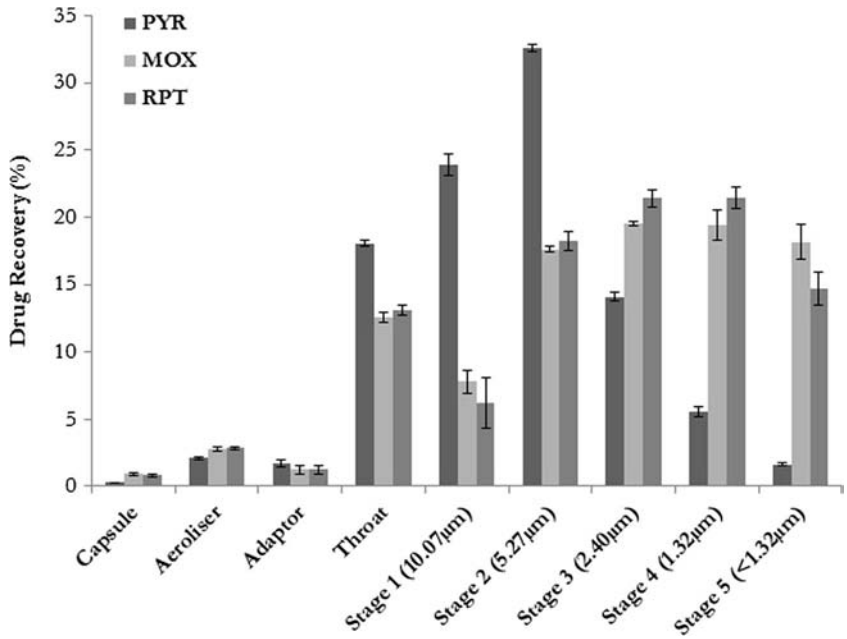


**Fig. 5** Aerosol deposition profile of RMP after 3 months storage (60% RH, 25°C), following MSLI dispersion at a flow rate of 100 L/min ($n = 3$). For each stage, levels of individual antibiotics are shown as a percentage of its own total actual recovered amount. Error bars show standard deviation.

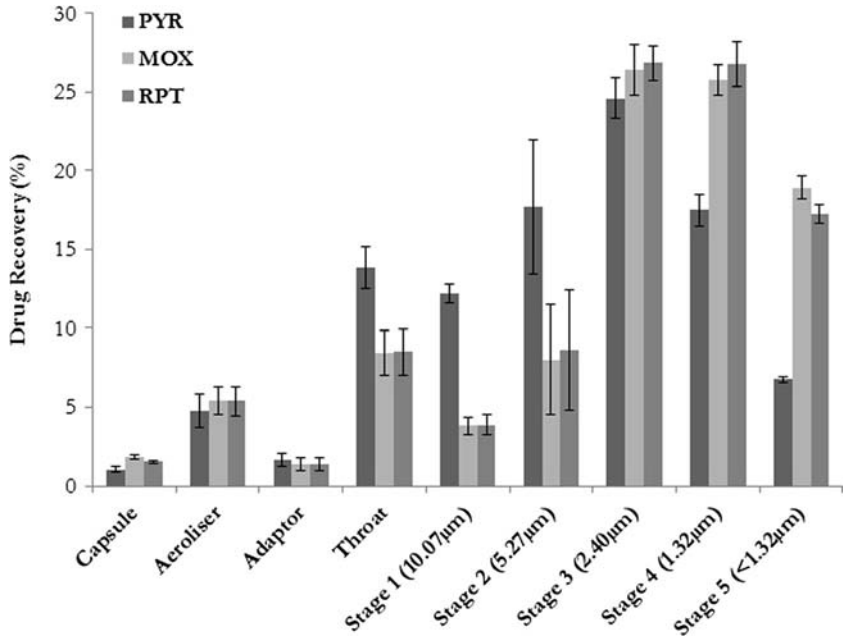


**Fig. 6** Aerosol deposition profile of RMPL after 3 months storage (60% RH, 25°C), following MSLI dispersion at a flow rate of 100 L/min ($n = 3$). For each stage, levels of individual antibiotics are shown as a percentage of its own total actual recovered amount. Error bars show standard deviation.

solubility of leucine in ethanol necessitates the use of water-ethanol co-solvents when spray drying with hydrophobic drugs (64). Learoyd *et al*. (2009) demonstrated an approach whereby a hydrophilic (tubertuline sulcate) and hydrophobic drug (beclometasone diproprionate), along with leucine, were successfully spray dried from a 30:70 v/v ethanol:water co-solvent system. Furthermore, a comprehensive study on leucine spray-dried from ethanol-water co-solvent systems, using budesonide as a model drug, an ethanol content of at least 50% v/v, was found to greatly improve the aerosol performance enhancing properties of leucine in the formulation (64). Thus leucine as an excipient in spray-dried formulations from ethanol co-solvent systems is well-established.

With regard to anti-tubercular drugs, capreomycin, a second-line antibiotic for TB, was co-spray-dried by Fiegel *et al*. (2008) with a range of leucine concentrations from a 50:50 v/v ethanol:water solution (53). A final capreomycin powder containing 20% w/w leucine was chosen for administration in a Phase 1 human clinical trial (65). Leucine concentrations for inhalable spray-dried formulations are generally between 10 and 20% w/w to impart an optimal balance between maximal drug loading and aerosol performance (53,63,66). Thus 10% w/w leucine was incorporated in our formulations, which were spray-dried from a co-solvent system of 50% v/v ethanol:water, to study its effects on powder aerosol performance and storage stability.

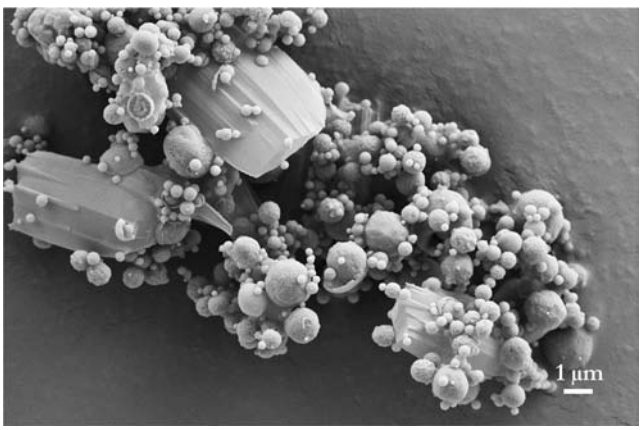


**Fig. 7** Scanning electron micrographs of pyrazinamide crystals present in RMP powder stored for 3 months at 60% RH (25°C).

The triple antibiotic powders have aerosol properties suitable for inhalation. The corrugated surfaces of RMPL may reduce inter-particle cohesion and result in superior aerosol dispersion as observed in its higher FPF (63.6±2.0%) (46,53,54). This is supported by the similarities in MMAD and GSD between RMP and RMPL (2.58±0.08 μm and 2.03±0.05 for RMP, and 2.51±0.06 μm and 1.94±0.05 for RMPL), but a 5.4% higher throat deposition with RMP associated with agglomerated particles, suggested that leucine increases aerosol performance by minimising agglomeration and aiding particle separation and delivery. Considering the aforementioned MMADs and the GSD of the powders, a wide drug distribution is expected throughout the entire lumen of the lung, from the upper regions, which may contain necrotic granulomas, down to the infected alveolar macrophages, making it suitable for treatment of active tuberculosis.

Inhaled delivery of the three drugs also directly enhances drug exposure at the pulmonary site of infection. In active TB,

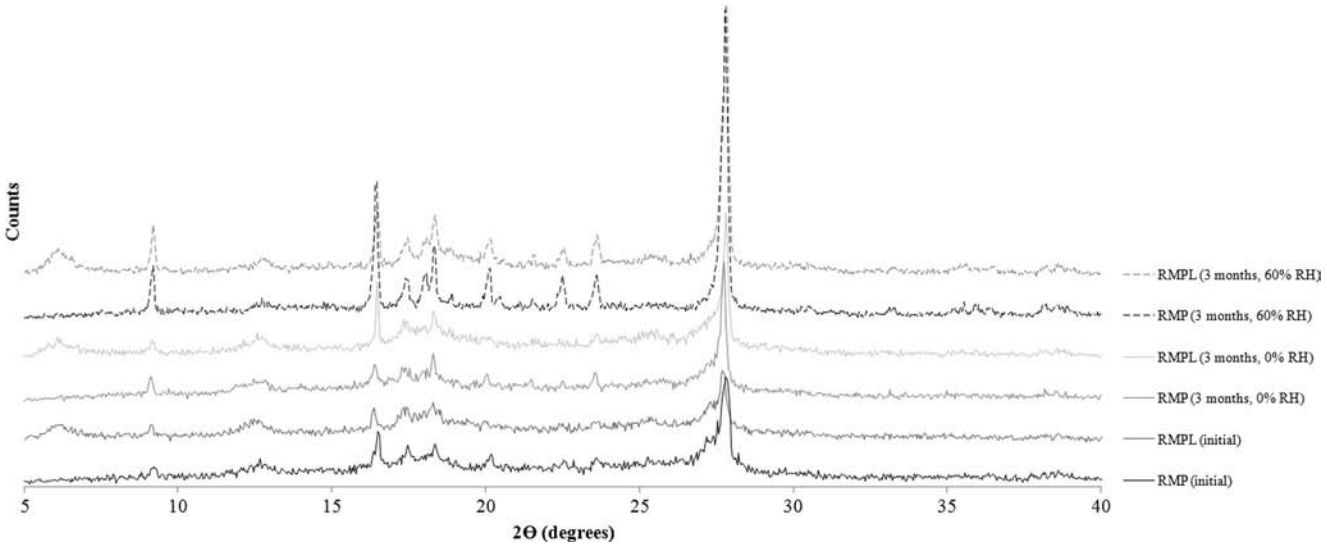


**Fig. 8** X-ray diffractograms for RMP and RMPL directly after spray drying and after storage at 0% and 60% RH for 3 months.

the primary goal is the elimination of extracellular bacillary population present in the lung cavity (12). Total drug exposure, rather than the maximum drug concentration achieved after dosing, is the primary determinant of antimicrobial activity for rifapentine, moxifloxacin and pyrazinamide (9,33,67–69). Our inhaled formulation is designed to maximise exposure of extracellular infection to the three aforementioned antibiotics.

The ratio of the three antibiotics in our inhaled formulation were chosen based on the current World Health Organisation approved doses for treating TB – specifically, rifapentine 600 mg, moxifloxacin 400 mg and pyrazinamide 1,500 mg (1,70). Although the US Food and Drug Administration has only approved rifapentine for intermittent dosing to treat active TB, the evidence for potential daily administration of rifapentine is sound as outlined earlier. Similarly, moxifloxacin is presently kept as a second-line treatment option, but there has been recent interest towards increasing the utilisation of moxifloxacin in first-line therapy and as a substitute for isoniazid (20,71). Pyrazinamide is one of the primary components of existing first-line anti-tubercular therapy and recent studies suggest increasing the current doses would enhance efficacy and clinical outcomes (36,38,69). Increasing the dose, however, is limited by a similar increase in hepatotoxicity. Leucine was incorporated in one of the formulations as it is a well-established enhancer of

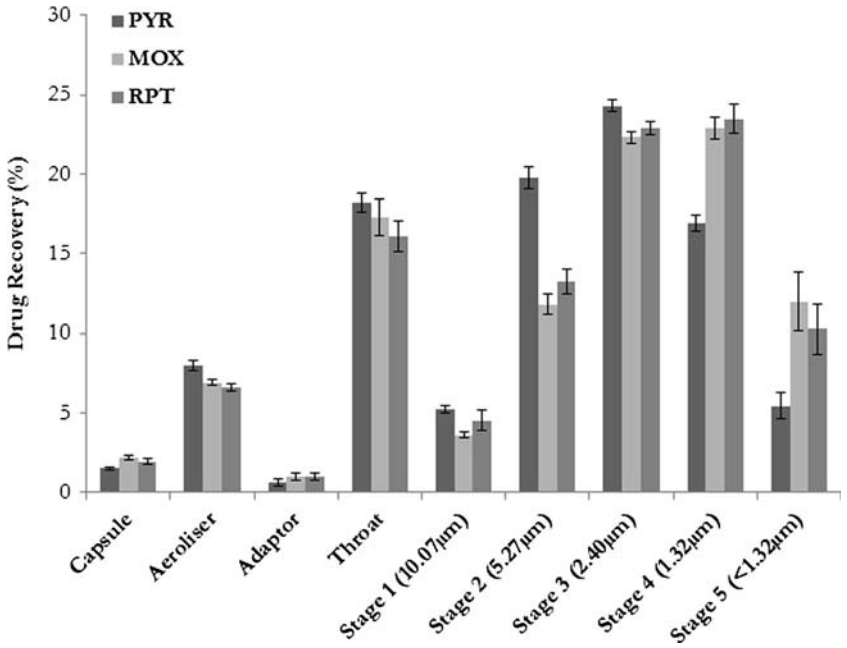


**Fig. 9** Aerosol deposition profile of RMP after 3 months storage (0% RH, 25°C), following MSLI dispersion at a flow rate of 100 L/min ($n = 3$). For each stage, levels of individual antibiotics are shown as a percentage of its own total actual recovered amount. Error bars show standard deviation.

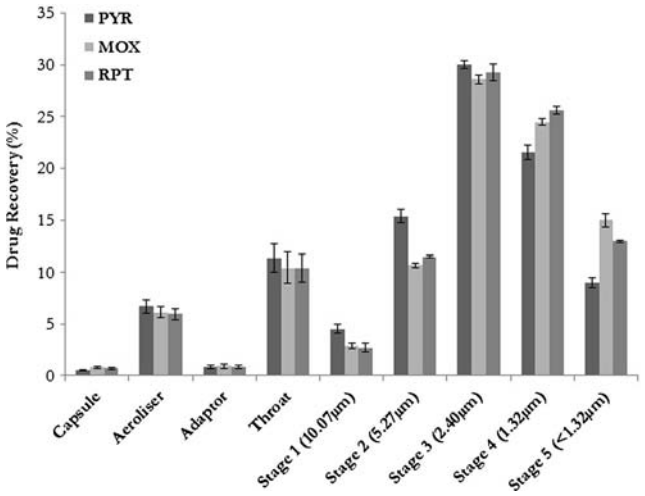


**Fig. 10** Aerosol deposition profile of RMPL after 3 months storage (0% RH, 25°C), following MSLI dispersion at a flow rate of 100 L/min ($n = 3$). For each stage, levels of individual antibiotics are shown as a percentage of its own total actual recovered amount. Error bars show standard deviation.

aerosolisation properties and chemical stability, as demonstrated with the current formulations (46,53,54). Particularly for pyrazinamide and rifapentine, there is a significant evidence for more efficacious anti-tubercular treatment associated with high antibiotic exposure achieved via inhaled delivery.

*In vitro* testing of the antibiotics against *M. tuberculosis* (H37Ra) showed the antimicrobial activity was not altered by spray-drying. Whilst we were unable to determine an MIC for pyrazinamide, its susceptibility testing is notoriously difficult and unpredictable because of significant influences from variations in pH as demonstrated by Zhang *et al*. (2002) (72). In a study using H37Ra, changes in the pH of the medium varied with inocula size, with a subsequent effect on the kill rate of pyrazinamide. Nonetheless, inclusion of pyrazinamide remained a useful control in demonstrating that it did not antagonise the activity of rifapentine and moxifloxacin.

Rifapentine composes a quarter of the antibiotic load in both RMP and RMPL, which explains their similar MICs of 4 ng/mL, which is four times the MIC concentration of rifapentine alone. This suggests neither of the other drugs had an antagonistic effect on rifapentine activity. The effect of moxifloxacin is masked by rifapentine as it is present at a lower concentration and has a significantly higher MIC. Although the resazurin assay did not present the three antibiotics as being synergistic *in vitro*, their additive efficacy as a combination, particularly the coupling of rifamycin and pyrazinamide, is well-established *in vivo* (2,6,36).

Combination antibiotic therapy in TB, such as an inhaled formulation of rifapentine, moxifloxacin and pyrazinamide, is essential for shortening treatment time and prevention of multi-drug resistance. In a multi-drug therapy, an antibiotic with a long half-life can have periods with concentrations above the minimum inhibitory concentration (MIC), whilst other compounds have concentrations below the MIC. The resulting virtual monotherapy with this long half-life drug results in rapid emergence of resistant bacteria (73). All three antibiotics in our proposed formulation (rifapentine, moxifloxacin and pyrazinamide) have similarly long plasma half-lives (10–18 hrs) (9,74,75), thereby maintaining sufficient drug levels to protect from resistance between dosing intervals for drug-susceptible tuberculosis. Synergistic resistance suppression has also been observed with the combination of a rifamycin and moxifloxacin (29,30).

Plasma half-life of anti-tubercular drugs has been shown to be extended when administered by inhalation. For example, endotracheal insufflation of spray-dried anti-tubercular agents – capreomycin and PA-824 – to *M. tuberculosis*-infected guinea pigs, doubled the drug plasma half-life relative to administration by IV/IM for capreomycin and oral for PA-824 (46,53,54). Extended antibiotic half-lives are expected to contribute to shorten treatment time by eliminating bacterial regrowth windows and perhaps also providing the option for intermittent rather than daily dosing.

Initial results from 3 months powder stability studies (Table III) indicate that RMP and RMPL are unsuited for storage at ambient conditions (25°C, 60% RH). Figure 7, an SEM image of RMP stored at 60% RH for 3 months at 25°C, clearly demonstrates the reason for this drug instability. Bulky pyrazinamide crystals, which were initially absent after spray-drying, are observed to have formed out of the original spherical particles at 3 months. The crystal growth is due to the instability of the crystalline δ polymorph pyrazinamide obtained from spray drying and reflected in the increased peak intensities for both powders in the XRPD analysis (Fig. 8) (40). The physical stability of pyrazinamide polymorphs is covered in detail by Pham *et al*. (2013). Additionally, moisture contributes to physical and chemical instability of pharmaceutical formulations, in this case primarily by accelerating the crystal growth of pyrazinamide (76). Although storage at 0% RH markedly improved the stability of RMP, only RMPL retained true stability after 3 months.

**Table IV** Combined Percentage Recoveries of Pyrazinamide, Moxifloxacin and Rifapentine from Stages 3 to 5 (particles < 5.27 μm) of the MSLI, for RMP and RMPL, Initially and After Three Months Storage at 0% RH

| | RMP | | RMPL | |
|---|---|---|---|---|
| Drug | Initial (%) | 3 months (%) | Initial (%) | 3 months (%) |
| Rifapentine | 60.0 ± 1.7 | 56.6 ± 1.9 | 67.7 ± 2.6 | 67.8 ± 0.9 |
| Moxifloxacin | 61.4 ± 2.1 | 57.2 ± 2.0 | 68.7 ± 0.2 | 68.1 ± 0.7 |
| Pyrazinamide | 52.0 ± 1.7 | 46.7 ± 1.0 | 62.3 ± 1.8 | 60.6 ± 1.0 |

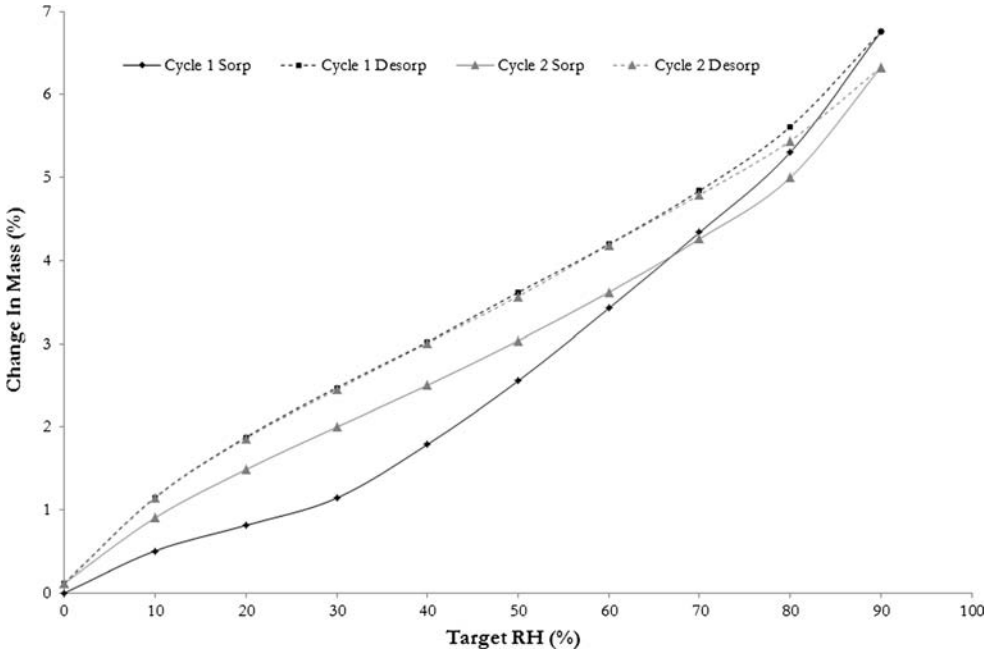


**Fig. 11** Moisture sorption isotherm for RMP with dual cycle humidity ramping from 0 to 90% RH.

Moisture sorption profiles for the two antibiotic-containing powders (Figs. 11 and 12) show crystallisation of pyrazinamide is an extended process. The minor decrease in mass gain in the RMPL formulation may be attributed to the hydrophobic nature of leucine. The slight differences in the hysteresis of the first and second moisture sorption profiles were associated with displacement of residual solvent from spray-drying. Reversible moisture sorption indicated phase transition of pyrazinamide did not occur in the short periods it was exposed to varying levels of moisture. Whilst the powders appeared to be physically stable in the short term, this was not maintained after 3 months of storage except under certain aforementioned conditions. An inhalable dry powder formulation containing rifapentine, moxifloxacin and pyrazinamide which is stabilised with leucine and appropriately protected from moisture, has shown chemical stability after 3 months, although long-term chemical integrity beyond this timepoint must be elucidated in ongoing studies.

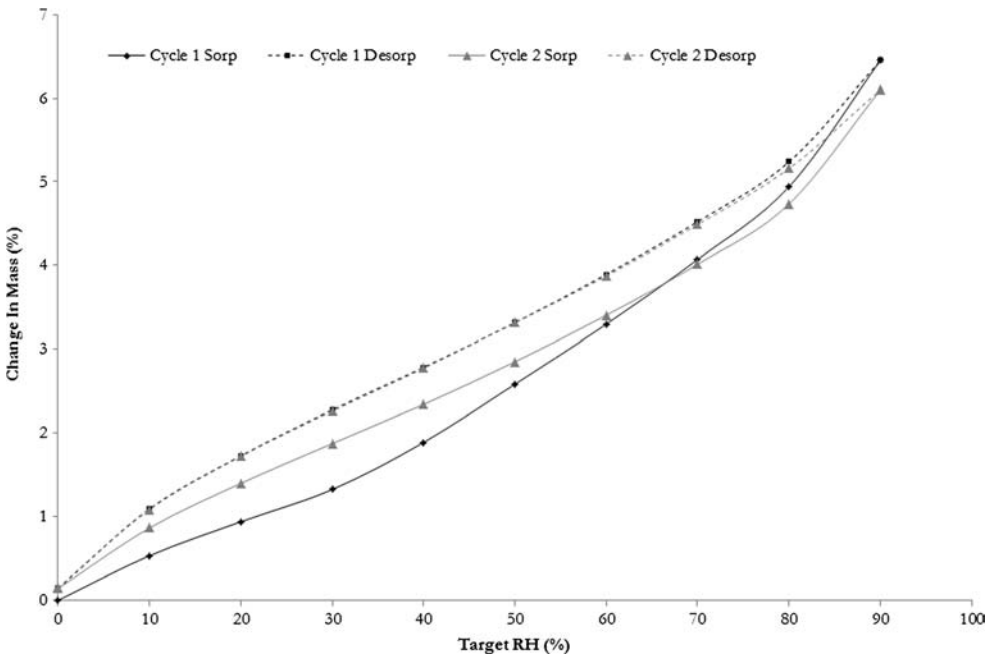


**Fig. 12** Moisture sorption isotherm for RMPL with dual cycle humidity ramping from 0 to 90% RH.

The thermal properties of RMP and RMPL in Fig. 4 show loss of residual solvent for spray dried moxifloxacin and rifapentine as broad endotherms (45 – 172 and 40 – 140°C, respectively). Melting followed by decomposition is evident for rifapentine as a baseline shift (161–173°C) and for moxifloxacin and pyrazinamide as sharp endothermic peaks at (188 and 257°C, respectively). RMP and RMPL exhibited altered thermal activity and interaction between the different components of the powders evident as multiple endothermic peaks from 112 to 188°C.

## CONCLUSIONS

The current work presents a novel inhalable co-spray-dried triple antibiotic formulation, with potentially improved efficacy for the treatment of TB. A formulation containing 10% w/w leucine to protect from moisture ingress and consequent enhancement of chemical stability was successfully formulated. This inhalable triple formulation, containing rifapentine, moxifloxacin and pyrazinamide is expected to maximise pulmonary antibiotic exposure at the primary site of infection. Furthermore, the use of these specific antibiotics has the potential to avoid drug-drug interactions, usually found with current therapies and shorten treatments times. *In vivo* pharmacokinetic studies in a murine model are currently underway.

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