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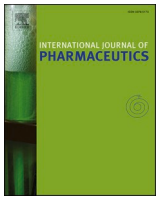

Synergistic combination of antimicrobial peptide and isoniazid as inhalable dry powder formulation against multi-drug resistant tuberculosis

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ABSTRACT

Multidrug-resistant tuberculosis (MDR-TB) has posed a serious threat to global public health, and antimicrobial peptides (AMPs) have emerged to be promising candidates to tackle this deadly infectious disease. Previous study has suggested that two AMPs, namely D-LAK120-A and D-LAK120-HP13, can potentiate the effect of isoniazid (INH) against mycobacteria. In this study, the strategy of combining INH and D-LAK peptide as a dry powder formulation for inhalation was explored. The antibacterial effect of INH and D-LAK combination was first evaluated on three MDR clinical isolates of *Mycobacteria tuberculosis* (*Mtb*). The minimum inhibitory concentrations (MICs) and fractional inhibitory concentration indexes (FICIs) were determined. The combination was synergistic against *Mtb* with FICIs ranged from 0.25 to 0.38. The INH and D-LAK peptide at 2:1 mole ratio (equivalent to 1: 10 mass ratio) was identified to be optimal. This ratio was adopted for the preparation of dry powder formulation for pulmonary delivery, with mannitol used as bulking excipient. Spherical particles with mass median aerodynamic diameter (MMAD) of around 5 μm were produced by spray drying. The aerosol performance of the spray dried powder was moderate, as evaluated by the Next Generation Impactor (NGI), with emitted fraction and fine particle fraction of above 70 % and 45 %, respectively. The circular dichroism spectra revealed that both D-LAK peptides retained their secondary structure after spray drying, and the antibacterial effect of the combination against the MDR *Mtb* clinical isolates was successfully preserved. The combination was found to be effective against MDR *Mtb* isolates with *KatG* or *InhA* mutations. Overall, the synergistic combination of INH with D-LAK peptide formulated as inhaled dry powder offers a new therapeutic approach against MDR-TB.

1. Introduction

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* (*Mtb*), which often leads to pulmonary infection. According to the official statistics announced by World Health Organization (WHO), TB killed approximately 1.6 million people in 2021 worldwide, ranking second as leading infectious killer after COVID-19 (WHO, 2022). Isoniazid, also known as isonicotinic acid hydrazide (INH), is a first-line anti-TB agent (Vilchèze and Jacobs, 2019). INH owns the advantages of superb bactericidal activity, narrow active bactericidal spectrum, low cost and high bioavailability (Unissa et al., 2016). Shortly after its discovery as anti-TB drug in 1952, resistance to INH became evident when it was used as a monotherapy (Fernandes et al., 2017; Unissa et al., 2016). WHO recommended a standard 6-month regimen consisting of INH and other three first-line anti-TB drugs, namely rifampicin, pyrazinamide and ethambutol, for drug susceptible TB (WHO, 2021). Nowadays, drug resistance becomes the foremost challenge to TB treatment. The frequency of resistant to INH was found to be significantly higher than to other anti-TB drugs and it is one of the most common types of resistance among TB patients (Nachega and Chaisson, 2003; Unissa et al., 2016). Treatment of INH-resistant TB with the WHO standard regimen resulted in treatment failure, relapse and epidemic of multi-drug resistance

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(MDR) TB, which refers to the resistance to at least INH and rifampicin (Gegia et al., 2017), both of them are the cornerstone of modern TB chemotherapy.

INH, a prodrug activated by the mycobacterial enzyme KatG inside the mycobacterial cell, has complex mechanism of action (Timmins and Deretic, 2006). Active INH products interfered many pathways pertaining to macromolecular synthesis, notably mycolic acid synthesis (Miesel et al., 1998). Lacking mycolic acid synthesis may affect the impermeability and acid fastness of the mycobacterial cell envelope (Judge et al., 2012; Miesel et al., 1998). The active INH products target different enzymes, such as enoyl acyl carrier protein (ACP) reductase (InhA) and beta-ketoacyl ACP synthase (KasA). The development of INH-resistance mainly related to the mutation in the *KatG* gene, followed by *InhA, ahpC, KasA* (Miesel et al., 1998; Slayden and Barry, 2000). In addition, INH can be inactivated by arylamine N-acetyltransferases (NATs) (Miesel et al., 1998).

Antimicrobial peptides (AMPs) serve as a novel alternative to anti-TB therapy especially against MDR-TB (Khusro et al., 2016; Silva et al., 2016). Cationic AMP can selectively interact and cross the cell envelope of microbes through more than one anionic target, leading to direct damage to bacterial plasma membranes or access to intracellular targets (Gutsmann, 2016). With their direct and rapid action, the incidence of resistance to AMPs could potentially be reduced (Bechinger and Gorr, 2017). AMPs could also serve as an immunostimulant with antimicrobial function due to their intracellular nucleic acid or protein inhibition activity (Diamond et al., 2009; Lai and Gallo, 2009). The two D-LAK peptides employed in this study, namely D-LAK120-A and D-LAK120-HP13, are cationic AMPs comprised of 25 D-enantiomer amino acid residues with α-helix conformation. Their D-conformation can withstand protease degradation with enhanced mycobacterial selectivity (Khara et al., 2016). Additionally, D-LAK120-HP13 contains proline residues, which disrupts the α-helix. The induction of proline residues may improve activity against Gram-negative bacteria and reduce hemolysis (Vermeer et al., 2012). Both D-LAK peptides were previously shown to exhibit detergent-like property that disrupts the colonies of *Mtb* strain H37Ra, inhibit the intracellular growth of *Mtb* in macrophage-like THP-1 cells, and potentiate the activity of the INH *in vitro* at non-toxic concentration (Lan et al., 2014). Another metabolic study revealed that D-LAK peptides influenced the remodeling of micromembrane in response to antibiotics and hence enhance their efficacy (Man et al., 2018). The proline containing D-LAK120-HP13 peptide also showed better antimycobacterial activity compared with the proline-free D-LAK120-A peptide, indicating a different mechanism against mycobacteria from Gram-negative bacteria (Man et al., 2018).

Pulmonary delivery of anti-TB agents is desirable for TB patients who typically manifest infectious lesion in the lungs. This non-invasive route of administration can achieve high drug concentration at the infection site, reduce systemic exposure and lessen the incidence of systemic adverse effects. Although INH can be administrated by oral or intramuscular route, it is not feasible to administer D-LAK peptides orally due to poor stability and absorption in the gastrointestinal tract. This study aimed to develop combined dry powder formulations of INH and D-LAK peptides as a potential new inhaled therapy against MDR-TB. The antibacterial activities of INH, D-LAK peptides and their combinations were first investigated on MDR clinical isolates of *Mtb*. Spray dried powder formulations of INH and D-LAK peptide combinations were prepared at their optimal ratio, and the powder were examined for their physicochemical properties and aerosol performance. The structural integrity of the D-LAK peptides and the *in vitro* antibacterial activity of the powder formulations were also evaluated.

2. Materials and methods

2.1. Materials

D-LAK120-A peptide (KKLALALAKKWLALAKKLALALAKK-NH_2) and D-LAK120-HP13 peptide (KKALAHALKKWLPALKKLAHALAKK-NH_2) with a purity over 80 % were purchased from EZBiolab (Parsippany, NJ, USA). INH, trifluoroacetic acid (TFA) and resazurin sodium salt were purchased from Sigma-Aldrich (Poole, UK). Mannitol (Pearlitol® 160) was obtained from Roquette (Lestrem, France). Middlebrook 7H9 Broth and Middlebrook oleic albumin dextrose catalase (OADC) were purchased from Becton Dickinson (Franklin Lakes, NJ, USA).

2.2. Mycobacterial strains

Three *Mtb* MDR clinical isolates namely HKU-14621, WC-286 and WC242 were employed in this study (**Supplementary Table S1**). All experiments involved *Mtb* were carried out in Block T Physical Containment Level 3 Laboratory, The University of Hong Kong.

2.3. Antibacterial assay of INH and D-LAK peptide combination

The minimum inhibitory concentration (MIC) of INH and D-LAK peptides (D-LAK120-A and D-LAK120-HP13) when used alone or in combination was measured on the three MDR strains. The interactions between INH and D-LAK peptides were evaluated using checkerboard assay (**Supplementary Fig. S1**) according to previous studies with modification (Man, 2019; Tam, 2020). A loopful of mycobacterial colonies was suspended in the double concentration of Middlebrook 7H9 broth medium with 20 % OADC and 0.4 % glycerol. The suspension was vortexed for 5 min with glass beads to obtain a homogeneous mycobacterial suspension. The suspension was adjusted to 1 McFarland turbidity standard by measuring the optical density (OD) at 600 nm using a spectrophotometer. Fifty microliter mycobacterial suspension and 50 μL of drug solution prepared in different concentrations were added to each well to achieve a final *Mtb* concentration of 1.5×10^8 CFU/well. Mycobacterial suspension without treatment and 7H9 broth medium were used as positive and negative control, respectively. After 7 days of incubation, 30 μL of resazurin dye (0.02 % w/v) was added to each well. The color change was inspected visually after a further 48 h of incubation. The lowest drug concentration that inhibited the growth of mycobacteria without colour change was defined as the MIC (Jadaun et al., 2007; Martin et al., 2003). Each INH/D-LAK peptide combination was examined on three *Mtb* strains in triplicate. The fractional inhibitory concentration index (FICI) was calculated to determine the interaction between INH and D-LAK peptides according to following formula:

$$\text{FIC index} = \text{FIC}_A + \text{FIC}_B = \frac{[A]\ in\ combination}{\text{MIC}_A} + \frac{[B]\ in\ combination}{\text{MIC}_B}$$

MIC_A and MIC_B are the MIC of INH and D-LAK peptides, respectively, when used as single agent; [A] and [B] are the MIC of INH and D-LAK peptides, respectively, when used in combination. The combinations are considered to be synergistic when the FICI $\leq$ 0.5; additive when 0.5 $<$ FICI $\leq$ 4; and antagonistic when FICI $>$ 4 (Lee et al., 2016).

2.4. Preparation of combined powder formulation of INH and D-LAK peptides by spray drying

The INH to D-LAK peptide molar ratio of 2:1 (equivalent to mass ratio of 1:10) was adopted for the preparation of spray dried powder. This ratio was chosen according to the antibacterial assay that demonstrated satisfactory growth inhibition of mycobacteria described in the

Table 1
The composition of SDIA and SDIB formulations.

Formulation	Peptide	Composition (% w/w)		
		INH	Peptide	Mannitol
SDIA SDIB	D-LAK120-A D-LAK120-HP13	1.0	10.0	89.0

previous section. Two formulations, namely SDIA and SDIB, were prepared at a mass ratio of 1:10:89 of INH: peptide: mannitol (Table 1). All three components were dissolved in distilled water to achieve a final solute concentration of 1 % (w/v). The solutions were spray dried using a laboratory spray dryer (B-290 Büchi Labortechnik AG, Flawil, Switzerland) with the following parameters: inlet temperature 80 °C, nitrogen atomisation flow rate 742L/h, aspiration 38 m^3/min, liquid feed rate 2.1 mL/min (Shao et al., 2023; Shao et al., 2021). A two-fluid spray nozzle with an internal diameter of 0.7 mm (Büchi Labortechnik, Flawil, Switzerland) was used and the spray dryer was operated at the open-loop configuration.

2.5. Production yield and drug content

The production yield was calculated as the mass of powder collected divided by the solid input. The content of INH and D-LAK peptides in the spray dried powder was quantified separately with high-performance liquid chromatography (HPLC, Agilent 1260 Infinity, Agilent Technologies, Santa Clara, USA). Weighed powder was dissolved in ultra-pure water to prepare a 5 mL solution which was filtered through a 0.45-μm nylon membrane filter. INH was quantified with an established HPLC method with minor modification (Ayyappan et al., 2011). An isocratic elution at 1 mL/min with phosphate buffer (pH 6.9) and methanol (95/5 v/v) was applied. A volume of 50 μL sample was injected and passed through the C18 column (250 mm × 4.6 mm, 5 μm, Agilent, USA) at room temperature. INH was detected by UV at 254 nm. The retention time of INH was around 8.5 min. The calibration curve was linear in the range between 5.0 μg/mL and 200 μg/mL (R^2 = 0.9998). The HPLC method for quantification of D-LAK peptide was adopted from previous studies (Kwok et al., 2015; Shao et al., 2023). The mobile phase composed of acetonitrile and ultra-pure water with 0.1 % TFA. A linear gradient from 20 % to 80 % acetonitrile was applied over 20 min at 1 mL/min. A C18 column (250 mm × 4.6 mm, 5 μm, VydacTM GraceTM, IL, USA) was employed and the peptide was detected by UV at wavelength 220 nm. The injection volume was 100 μL and the analysis was performed at room temperature. The retention time of D-LAK peptides was around 9.85 min. Linearity for D-LAK peptides was demonstrated between 8.0 μg/mL and 200 μg/mL (R^2 = 0.9998). The method of mannitol quantification was described in section 2.7.

2.6. Morphology study

The morphology of SDIA and SDIB powders was evaluated by scanning electron microscopy (SEM, Hitachi S-4800, Hitachi High-technologies Crop., Tokyo, Japan) at 5.0 kV and 10.0 kV. The powder was sprinkled on the adhesive carbon discs with excess powders removed, and sputter-coated with approximately 13 nm gold–palladium alloy in two cycles (60 s each) using a sputter coater (Q150T Plus Turbomolecular pumped coater, Quorum, UK) to aid charge dissipation during imaging.

2.7. Aerosol performance

The Next Generations Impactor (NGI) (Copley Scientific Limited. Nottingham, UK) experiment was conducted to evaluate aerosol performance of SDIA and SDIB powders. Hydroxypropyl methylcellulose (HPMC) capsules were loaded with 5 mg of powder, and Breezhaler® (Novartis Pharmaceuticals, Hong Kong) was used for aerosolisation. The powders were dispersed at an airflow rate of 90 L/min for the duration of 2.7 s (Liao et al., 2020). After dispersion, the powders deposited in capsule, inhaler, adaptor, throat and each stage of NGI were dissolved in 4 mL of distilled water and assayed by HPLC with a refractive index detector (G1362A, Agilent Technologies, Santa Clara, USA). Mannitol which was the major component in the formulations was quantified. A volume of 50 μL filtered solution was injected and passed through two ion-exchange ligand-change columns (Agilent Hi-Plex Ca, 7.7 × 50 mm, 8 μm; Agilent Technologies) at 75 ℃ with a flow rate of 0.6 mL/min. The total amount of powder recovered from the dispersion was referred as the recovered dose. Emitted fraction (EF) was defined as the amount of powder that exited the inhaler with respect to the recovered dose. Fine particle fraction (FPF) referred to the amount of powder with an aerodynamic diameter less than 5.0 μm (calculated by interpolation) with respect to the recovered dose. The mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) were calculated by a linear plot of cumulative mass percentage against the logarithm of the cut-off diameters.

2.8. Thermogravimetric analysis (TGA)

The residual moisture of spray dried powders was assessed by thermogravimetric analysis (TGA) (TGA550, TA Instruments, New Castle, DE, USA). The powder was heated from 25 ℃ to 160℃ at 10 ℃/min with 60 mL/min nitrogen purge. The residual moisture was determined by the weight loss of powder due to the evaporation of water.

2.9. Differential scanning calorimetry (DSC)

The thermal response profiles of the spray dried powders and raw materials were measured by differential scanning calorimetry (DSC) (Q1000, TA Instruments, New Castle, DE, USA). The powder was heated from 25 ℃ to 250 ℃ at 10 ℃/min under 50 mL/min nitrogen purge. The thermogram of each sample was plotted.

2.10. Antibacterial assay of SDIA and SDIB formulations

The antibacterial activity of SDIA and SDIB powder formulations was evaluated by measuring their MICs on three *Mtb* strains. Each powder formulation was reconstituted with ultra-pure water to obtain an aqueous stock solution. The reconstituted SDIA and SDIB formulations were prepared at various concentrations in two-fold dilution, ranging from 2 μM to 256 μM of INH (except for WC-242 strain in which the formulations were tested at concentrations of INH from 4 μM to 512 μM). The preparation of inoculum, incubation and resazurin dye method were the same as described in section 2.3. The MICs and FICIs of each spray dried powder formulations were obtained.

2.11. Circular dichroism (CD)

The secondary structure of peptide was examined by circular dichroism (CD). SDIA and SDIB powders were dissolved in phosphate-buffered saline (PBS) to achieve a final peptide concentration of 0.15 mg/mL. The two D-LAK peptides were also prepared in PBS at the same concentration as controls for comparison. The CD spectra were acquired using a JASCO J-1500CD spectrophotometer and recorded in quartz cuvettes with a path length of 10 mm and a volume of 300 μL. The scan range was set from 190 nm to 240 nm, with a scan speed of 200 nm/min, a spectral bandwidth of 1 nm, and 4 accumulations for each sample. The digital integration time was set to 1 s.

3. Results

3.1. Antibacterial assay of INH and D-LAK peptide combination

The antibacterial activities of INH and D-LAK peptide combinations were examined by measuring the MICs on MDR *Mtb* strains and their synergistic effects were evaluated by their FICIs (Fig. 1). The MICs of INH on MDR clinical isolates were all higher than 256 μM (the highest concentration tested) when it was used alone Supplementary Fig. S2. D-LAK peptide alone did not inhibit the growth of *Mtb* and their MICs were also higher than 256 μM. However, when INH and D-LAK peptide were used in combination, the three MDR isolates became susceptible to INH. There was a synergistic effect of INH and D-LAK peptides combination

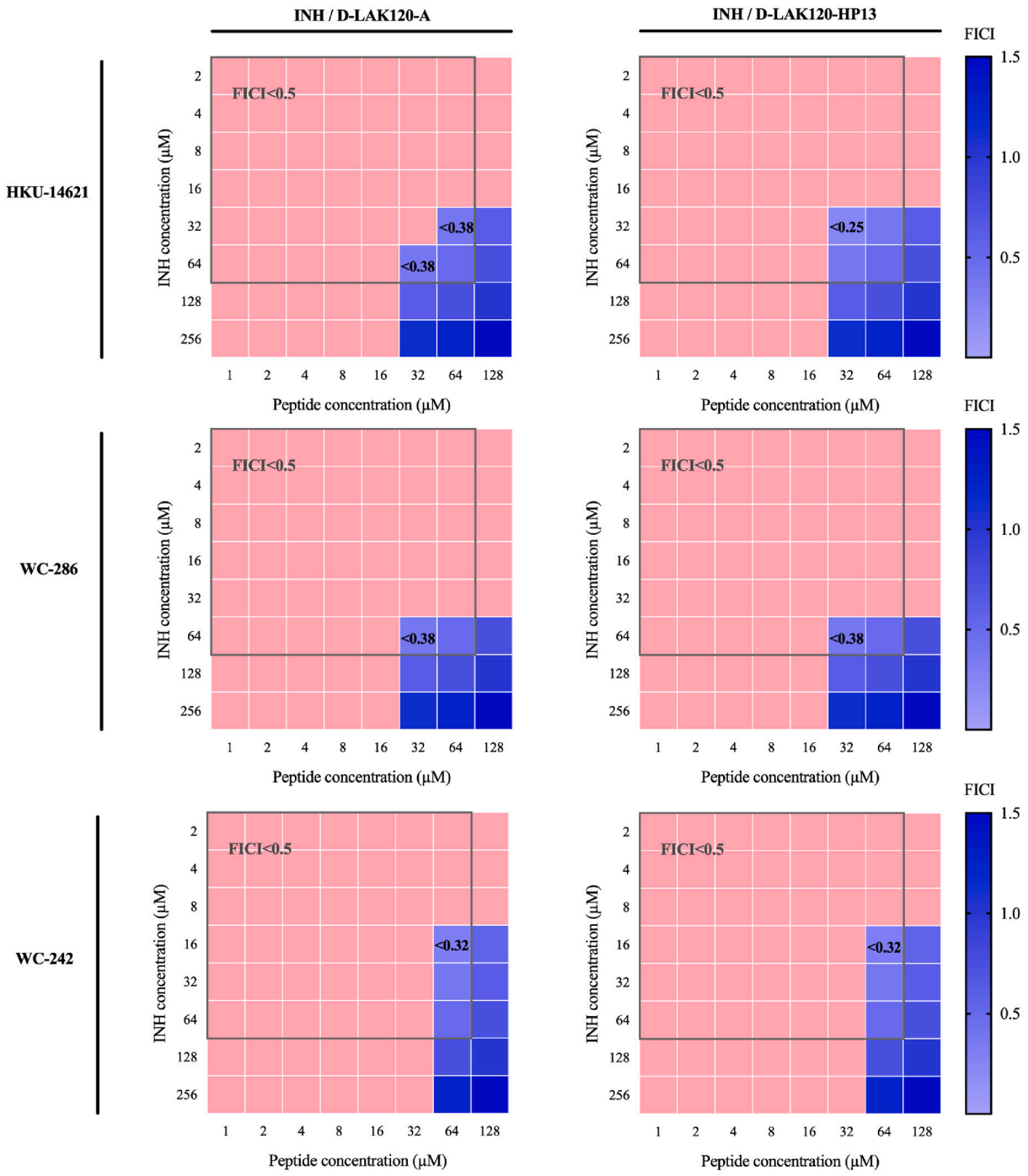

Fig. 1. The antibacterial activity of INH and D-LAK peptide combination using checkerboard assay. Each well contained different concentrations of INH and peptides. Pink colour represents wells with colour change of resazurin dye, indicating mycobacteria growth; blue colour represents wells without colour change, indicating no growth of mycobacteria. The wells with minimum FICI against the specific strain were labelled with the FICI values. The wells with FICI < 0.5 were highlighted with a grey square frame. The most representative data of the three repeats are shown. (For complete data set, please refer to Supplementary Fig. S2).

Table 2
The production yield, drug content and residual moisture of SDIA and SDIB spray dried formulations. Data of the measured percentage were presented as mean ± standard deviation (n = 3).

Formulation	Production yield (%)	Measured (%)			Residual moisture (%)
		INH	Peptide	Mannitol	
SDIA	73.86	0.85 % ± 0.02 %	8.55 % ± 0.21 %	90.51 ± 1.82	1.40
SDIB	70.65	0.87 % ± 0.02 %	10.80 % ± 0.28 %	88.17 ± 2.83	1.10

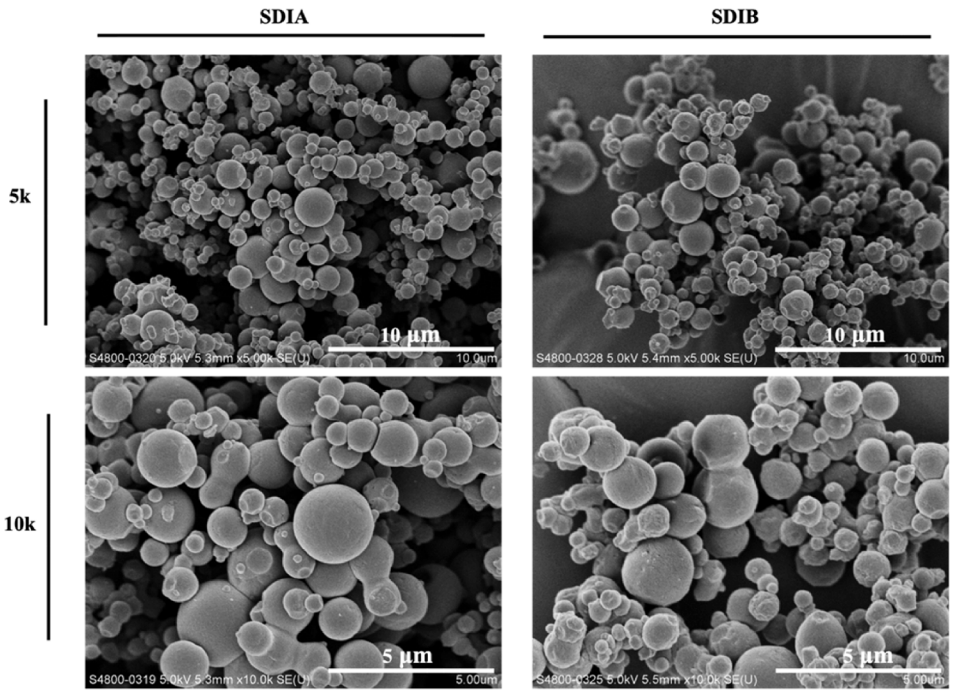

Fig. 2. The scanning electron microscopy (SEM) images of spray dried powder of SDIA and SDIB formulations at 5 k and 10 k magnification.

Table 3
Aerosol performance of SDIA and SDIB spray dried formulations evaluated by Next Generation Impactor (NGI). Data was presented as mean ± standard deviation (n = 3). MMAD, mass median aerodynamic diameter; GSD, geometric standard deviation.

Formulation	Emitted Fraction (%)	Fine Particle Fraction (%)	MMAD (μm)	GSD
SDIA	72.87 ± 2.01	46.67 ± 2.76	4.99 ± 0.20	2.51 ± 0.23
SDIB	74.33 ± 3.41	47.19 ± 2.20	4.89 ± 0.24	2.37 ± 0.10

on all MDR strains tested, in which the FICIs were below 0.5. D-LAK120-A and D-LAK120-HP13 showed similar effect. When designing the combined dry powder formulation, the ratio between INH and D-LAK peptides that demonstrated synergistic effect against MDR strains was the primary consideration. The INH to D-LAK peptide ratio of 2:1 (molar ratio) was selected for the development of dry powder formulation. because it showed the most consistent inhibitory activity in both D-LAK120-A and D-LAK120-HP13 peptides against MDR strains.

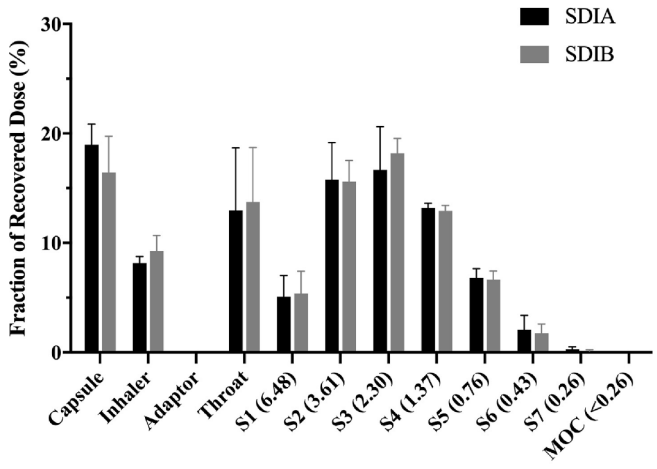

Fig. 3. The Inter-stage distribution of INH/peptide co-spray dried powder formulations (NGI, Breezhaler®, 90 L/min). S1 to S7 represented the first seven stages of NGI. The corresponding lower cut-off diameter (in μm) was given in parenthesis. MOC represented micro-orifice collector. Data were presented as mean ± standard deviation (n = 3).

3.2. Production yield, drug content and residual moisture

Spray drying was used to prepare the combined dry powder formulations, SDIA and SDIB. The current spray drying condition resulted in an outlet temperature between 44 and 48 ℃. Both SDIA and SDIB powder formulations showed satisfactory production yields of over 70 % (Table 2). The measured content of INH, D-LAK peptides and mannitol approximated to the theoretical values. The residual moisture of the two spray dried formulations was less than 1.5 %, suggesting that the spray drying condition was suitable for preparing dry powder of INH and D-LAK peptide combination.

3.3. Particle morphology and aerosol performance

The SEM images of SDIA and SDIB powder were obtained at 5 k and 10 k magnification (Fig. 2). In general, the particles were spherical in shape with smooth surface. Most of the particles ranged between 1 and 3 μm. No notable difference between the two formulations was observed. The aerosol performance of SDIA and SDIB powders was evaluated with NGI coupled with Breezhaler® operated at a flow rate of 90 L/min (Table 3). Both spray dried formulations showed moderate aerosolisation properties with EF above 70 %, FPF above 45 %, and MMAD below 5 μm. However, the inter-stage distribution of the spray dried formulations showed that over 15 % of powder remained in the capsule after dispersion, and nearly 10 % of powder stayed in the inhaler device (Fig. 3).

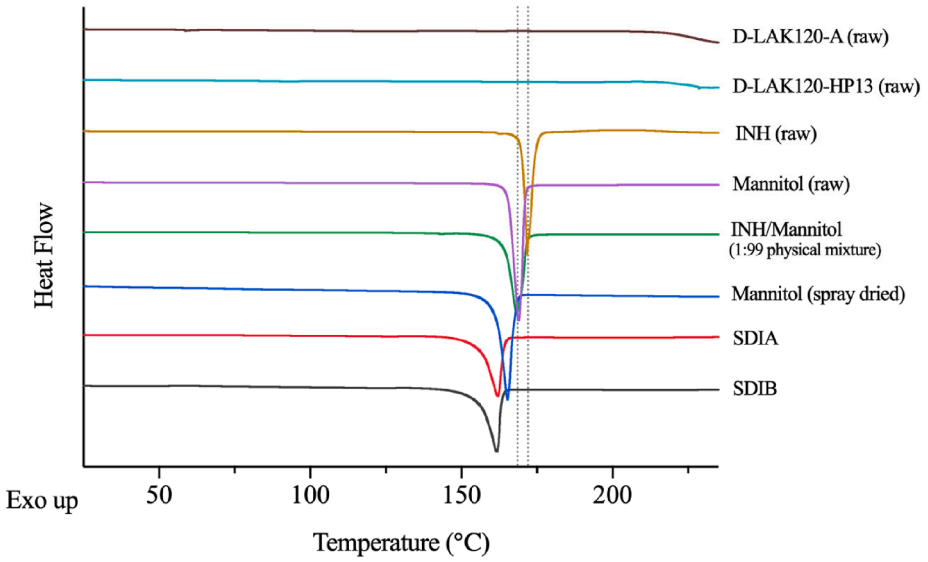

Fig. 4. DSC thermogram of SDIA and SDIB formulations. Negative peak represents endothermic events. The dotted line represents 166℃ and 172℃. Unformulated peptide (raw), unformulated INH (raw), unformulated mannitol (raw), INH/mannitol (1:99 w/w physical mixture) and spray dried mannitol, were included as controls for comparison.

3.4. Thermoanalysis

The thermal profiles of SDIA and SDIB powders as well as the raw materials were obtained by DSC (Fig. 4). A characteristic endothermic peak at approximately 172 ℃ was observed in raw INH, which corresponded to the melting point of INH. Raw D-LAK120-A and D-LAK120-HP13 peptide did not show any peak between 25 ℃ to 250 ℃, indicating that they could be amorphous. Mannitol had an endothermic peak at around 166 ℃, which corresponded to its melting point. The endothermic peak of mannitol after spray drying shifted due to melting point depression caused by the smaller particles size of mannitol (Rim and Runt, 1984). The SDIA and SDIB spray dried formulations showed only one endothermic peak which was close to the spray dried mannitol. The presence of INH and D-LAK peptide in SDIA and SDIB reduced the melting point of mannitol. Both SDIA and SDIB dry powder did not show the characteristic endothermic peak of INH melting point. To examine whether the absence of this peak was due to the low content of INH in the formulation (1 % w/w) that render it undetectable, or the transition of INH from crystalline to amorphous during spray drying, a sample containing the physical mixture of INH and mannitol at a mass ratio of 1 to 99 was measured. The lack of INH peak suggested that the low content of INH was indeed the explanation.

3.5. Secondary structure of D-LAK peptides in SDIA and SDIB formulations

To evaluate the effects of spray drying on the secondary structures of D-LAK peptides, the CD spectra of D-LAK peptides with or without going through spray drying were compared (Fig. 5). D-LAK120-A peptide showed a strong ellipticity at 205 nm (Fig. 5A), while D-LAK120-HP13 peptide provided a strong positive ellipticity at around 198 nm (Fig. 5B), indicating that both D-LAK peptides exhibit α-helix conformation. Notably, D-LAK120-A displayed an additional positive band at around 220 nm as the peptide without proline kink exhibited greater α-helix conformation. For both D-LAK peptides, the CD spectra of the unformulated peptide superimposed almost perfectly with that of the spray dried powder formulations, suggesting no significant change in the overall secondary structure after the spray drying process.

3.6. Antibacterial effect of SDIA and SDIB formulations

To investigate the antibacterial effect of SDIA and SDIB formulations

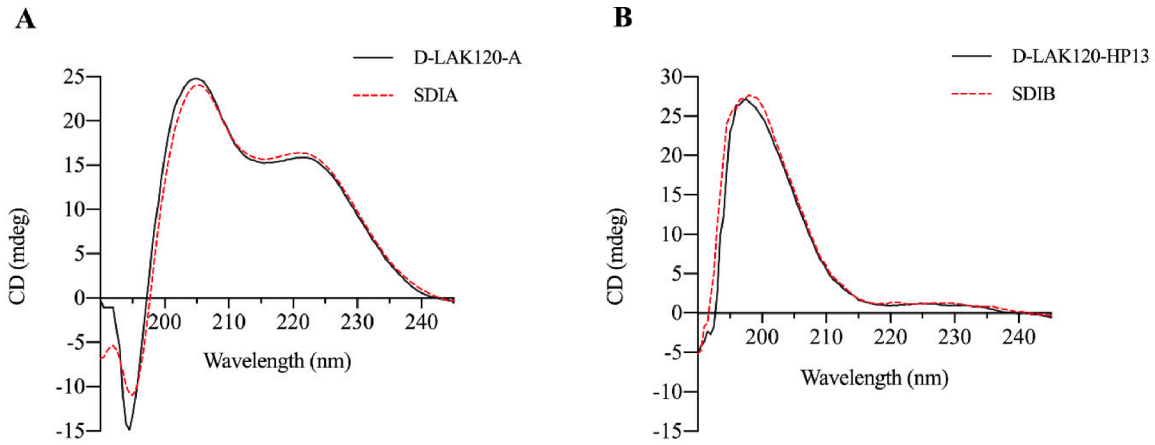

Fig. 5. Circular dichroism (CD) spectra of SDIA and SDIB formulations containing D-LAK120-A (A) and D-LAK120-HP13 (B). The unformulated peptides were included as controls for comparison.

Table 4
The MICs and FICIs of SDIA and SDIB formulations (n = 3, data of three repeats were identical).

Strains	MIC (μM)			Formulation SDIA			Formulation SDIB		
	INH	PA	PB	MIC (μM)		FICI [a]	MIC (μM)		FICI [a]
				INH	PA		INH	PB	
HKU-14621	>256	>256	>256	64	32.48	<0.38	64	31.61	<0.38
WC-286	>256	>256	>256	64	32.48	<0.38	64	31.61	<0.38
WC-242	>512[b]	>256	>256	128	64.96	<0.51	64	31.61	<0.25

PA: D-LAK120-A; PB: D-LAK120-HP13.
[a] : As the MICs of INH or peptide used alone were higher than the highest tested concentration, the FICI cannot be calculated as a specific value. The FICI was calculated with the assumption that the MICs of INH or peptide alone are 256 μM. The actual FICI should be smaller than the calculated value.
[b] : Higher INH concentrations were tested on WC-242.

against *Mtb*, the powder was reconstituted at various concentrations and added to the three MDR *Mtb* strains, and their MICs and FICIs were measured (Table 4). In general, the antibacterial activities of SDIA and SDIB formulations were successfully preserved. In HKU-14621 and WC-286 strains, the SDIA and SDIB formulations showed similar MICs compared with the corresponding INH and D-LAK combination before formulation. In WC-242 strain, the optimal molar ratio of INH to D-LAK peptide to achieve antibacterial effect was 1: 4 (i.e., 1: 80 mass ratio) according to the combination study, instead of 2:1 (i.e. 1: 10 mass ratio) employed in SDIA and SDIB formulations. As a result, both powder formulations had higher MIC of INH than that obtained in the combination study, which could be interpreted as the insufficient amount of peptide used in the formulation. The MIC of INH in SDIA was higher than that observed in SDIB, which could be partially explained by the lower peptide percentage measured in the former formulation (Table 2). Although SDIA formulation only displayed modest antibacterial activity against WC-242 strain with FICI barely above 0.5, both SDIA and SDIB formulations showed FICI of less than 0.5 in other two *Mtb* strains, indicating their capability to exert synergistic effect in some MDR-TB strains.

4. Discussion

INH resistance is one of the most common types of *Mtb* resistance for decades with the overall prevalence between 10.7 % and 27.2 % (Sulis and Pai, 2020). The resistance to INH resulted in lowering the treatment success rate (especially the 9-month standard regimen) with the higher risks of acquiring resistance to other anti-TB drugs and developing MDR-TB (Jhun and Koh, 2020). Bacterial genetics has an impact on the transmission of INH-resistant *Mtb* (Gagneux et al., 2006), with 40 to 95 % of INH-resistant isolates have mutation in KatG (Hazbón et al., 2006). *KatG* gene encodes the catalase-peroxidase enzyme that can activate the prodrug INH to an active intermediate (Wengenack et al., 1998). It was widely acknowledged that the stable oxidation products of INH do not demonstrate antibacterial activity while the active intermediate contributes to enzyme inhibition involving mycolic acid synthesis (Johnsson and Schultz, 1994). As a prodrug requires activation, the presence of functional KatG is crucial for INH to exert its antibacterial effect. Around 75 to 90 % of *KatG* mutations of INH-resistant *Mtb* are located in codon 315, which decreases INH activation without abolishing catalase-peroxidase activity (Hazbón et al., 2006; Kapetanaki et al., 2005). In KatG with S315T mutation, the serine at amino acid residue 315 is replaced with threonine and this mutation confers an 80-fold increase in the MIC of INH (Musser et al., 1996). Here, both HKU-14621 and WC-286 *Mtb* clinical isolates displayed the S315 *KatG* mutation, suggesting that these isolates would have poor ability to recognize INH as a substrate (Wengenack et al., 1998). The presence of D-LAK peptide reduced the MICs of INH against HKU-14621 and WC-286 strains. In contrast, WC-242 which is another MDR clinical isolate of *Mtb* with wild-type *KatG* gene, showed a lower MIC of INH in the presence of D-LAK peptides. The results indicate that the genotype in *KatG* may influence the MIC of INH when combined with D-LAK peptides, but the mechanism requires further investigations.

Compared with *KatG* mutation which is more common in the MDR-TB, mutations in the *InhA* promoter are significantly more common in INH mono-resistant strains (Hazbón et al., 2006). Unlike *KatG* gene mutations which are responsible for high-level INH resistance in *Mtb*, mutations in *InhA* are associated with low-level resistance to INH (Heym et al., 1994; Somoskovi et al., 2001). InhA is a nicotinamide adenine dinucleotide (NADH)-dependent ACP reductase that catalyses the growing fatty acid chain linked to ACP. The reduction catalysed by InhA was utilised by mycobacteria to synthesise mycolic acid, which is a vital component of mycobacterial cell wall. During this process, the NADH and long chain acyl-ACP substrates are competitively bound to inhA. Most of the wild type inhA demonstrated a preference for NADH binding. This NADH-bound form of InhA can be attacked by activated INH, resulting in the inhibition of enzyme and the prevention of mycolic acid synthesis (Guo et al., 2006; Rozwarski et al., 1998). Mutation in *InhA* such as S94A and I194T (common mutation in *inhA* promoter) (Lempens et al., 2018) decreases the binding affinity of InhA to NADH, which promotes the acyl-ACP substrates binding to inhA before NADH and protects most of the enzymes from activated INH (Banerjee et al., 1994; Munir et al., 2019). WC-242 isolate displayed *inhA* gene mutation (I194T). The presence of D-LAK peptides was able to lower the MIC of INH against WC-242 isolate. Although the lowest MIC of INH achieved (16 μM) when used in combination with D-LAK peptides was lower in WC-242 than in the *KatG* mutated isolates (32 to 64 μM in both HKU-14621 and WC-286), a much higher concentration of D-LAK peptide is needed for antibacterial activity. Here, a fixed molar ratio of 2:1 of INH to D-LAK peptide was employed in both SDIA and SDIB powder formulations. This ratio was favourable for the two MDR *Mtb* strains with *KatG* mutation. Even this ratio was suboptimal of WC-242 strain with *inhA* mutation, SDIB still demonstrated good anti-TB activity and synergy between INH and D-LAK peptides. Formulation with a fixed ratio of INH to D-LAK peptides provide a potential therapeutic strategy for combating MDR-TB. Although a higher amount of peptides could be utilised for a wider applicability on different MDR-TB strains, due to their high membrane activity, the *in vivo* safety profile of the peptides and the peptide/INH combinations, especially in the respiratory system, must be carefully evaluated.

In our previous study, it was observed that D-LAK peptides could disrupt the heavily clumped mycobacteria (Lan et al., 2014). One hypothesis is that D-LAK peptides could potentiate the effect of INH by interfering the hydrophobic cell wall interaction and thereby increasing the permeability of the hydrophilic INH across mycobacterial cell walls. It was well-known that the highly lipid-rich mycobacterial membrane could effectively reduce permeability and therefore developing resistance to anti-TB drugs (Méndez-Samperio, 2008). Other AMPs such as human beta-defensin-1 (HBD-1), protegrin-1 (PG-1) and human neutrophil peptide-1 also demonstrated similar mechanism of potentiating anti-TB drugs' effect (Fattorini et al., 2004; Kalita et al., 2004). High-dose INH have been included in the shorter-course MDR-TB regimen recommended by WHO (WHO, 2022). It was found that high-dose INH demonstrated comparable bactericidal activity on cases of

low-level INH resistance (that always conferred by *InhA* mutations) compared with standard dose among drug-susceptible cases (Dooley et al., 2020; Gausi et al., 2021; Wasserman and Furin, 2020). It was generally considered that high-dose INH was ineffective in strains with *KatG* mutation which was associated with high-level resistance of INH. However, some studies supported that high-dose INH might have clinical efficacy for these mutation cases (Rieder and Van Deun, 2017; Walsh et al., 2019). The mechanism for clinical benefit of high-dose INH might be associated with the early bactericidal activity (EBA) of INH (Wasserman and Furin, 2020). As INH displays dose-dependent EBA, higher dose causes higher exposures, translates into efficacy and overcomes low to intermediate level of INH resistance (Wasserman and Furin, 2020). Here, the presence of D-LAK peptides possibly increases drug permeability and improves INH concentration in mycobacterial cells, displaying similar effect as high-dose INH. Further investigation on the mechanism of the synergistic effect between INH and D-LAK peptides against *KatG* and *InhA* mutated *Mtb* is warranted.

In the production of spray dried powder formulation, preserving structural integrity and biological activity of macromolecules is a challenge as the molecules are inevitably exposed to thermal, shear and interfacial stresses during spray drying. Both D-LAK120-A and D-LAK120-HP13 are linear peptides, their secondary structures may influence their affinity to the mycobacterial membrane, therefore affecting their antimicrobial effect. The CD spectra showed that the secondary structure of D-LAK peptides was successfully preserved, which could be attributed to the relatively low inlet temperature used in the current spray drying condition. The anti-TB activity of the powder formulations also demonstrated good biological activity of the drugs after spray drying. In terms of the aerosol performance, both SDIA and SDIB powder formulations showed a modest aerosolisation property with FPF over 45 %, and EF just below 75 %. However, more than 15 % powder was found to be remained in the capsule, indicating insufficient powder de-agglomeration during the dispersion. De-agglomeration of dry powder highly depends on powder properties (Chew and Chan, 2002). Since all three components in the powder formulations (i.e. INH, D-LAK peptide and mannitol) are hydrophilic, the spray dried particles are expected to exhibit strong cohesive force, which in turn hinders powder deagglomeration and emission significantly (Bosquillon et al., 2001; French et al., 1996). As both SDIA and SDIB adopted the same formulation (i.e. same excipient, same INH to peptide ratio, and same drugs to excipient ratio) and same production method, it was not surprising that their aerosol performance was similar. To enhance the aerosolisation property, dispersion enhancer such as leucine can be incorporated as an additional excipient. The hydrophobic nature of this amino acid allows it to be enriched on the particle surface during spray drying, thereby reducing particle cohesion without increasing particle size substantially and maintaining its suitability for pulmonary delivery (Chow et al., 2017; Sou et al., 2011).

Overall, the combination of INH and D-LAK peptides was formulated as spray dried powder suitable for inhalation. Using resazurin assay, the synergistic antibacterial activity of the combination was demonstrated in clinical isolates of MDR-TB. While resazurin assay is a simple and rapid method for detecting drug susceptibility in *Mtb* by assessing the colour change of the dye, it is a qualitative analysis. Conventional culture methods using agar-based media such as Lowenstein-Jensen medium, as well as *ex vivo* and *in vivo* studies that examine the intracellular killing of *Mtb* will be performed in future study to reinforce current findings (Horvati et al., 2021; Jadaun et al., 2007; Sirgel et al., 2009).

5. Conclusions

Inhalable dry powder formulations containing INH and D-LAK peptides were successfully prepared by spray drying. Both SDIA and SDIB formulations demonstrated synergistic effect between INH and D-LAK peptide against clinical isolates of MDR-TB. With mannitol as bulking excipient, the powder formulations exhibited good production yield and moderate aerosol performance. The aerosol performance of the spray dried formulations could be further enhanced by the incorporation of dispersion enhancer such as hydrophobic leucine to improve powder de-agglomeration. The mutation of *KatG* and *InhA* genes of *Mtb* clinical isolates influenced the synergistic effect and hence the optimal ratio between INH and D-LAK peptides. Nonetheless, the current combination was effective against MDR *Mtb* isolates with either mutation. To improve the combined formulation with a broader application on different MDR-TB strains, further studies of the combination on other clinical isolates with different gene mutations are imperative.

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Author contribution

Conceptualization: J.K.W.L. and Z.S; Methodology: Z.S., K.K.T., E.C.Y.W., A.J.M., W.C.Y. and J.K.W.L.; Formal analysis: Z.S., A.P.K.V., E.C.Y.W., S.F.C. and J.K.W.L.; Investigation: Z.S., K.K.T., A.P.K.V. and E.C.Y.W.; Resources. J.K.W.L., E.C.Y.W., A.J.M., S.F.C. and W.C.Y.; Writing-Original draft: Z.S. and J.K.W.L.; Writing-Review & Editing: J.K.W.L.; Validation: J.K.W.L.; Supervision: J.K.W.L.; Funding acquisition: J.K.W.L.. All authors have read and agreed to the published version of the manuscript.

CRediT authorship contribution statement

Zitong Shao: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Kingsley King-Gee Tam:** Investigation, Methodology. **V.P.K. Achalla:** Formal analysis, Investigation. **Esther C.Y. Woon:** Formal analysis, Investigation, Methodology, Resources. **A. James Mason:** Methodology, Resources. **Shing Fung Chow:** Formal analysis, Resources. **Wing Cheong Yam:** Methodology, Resources. **Jenny K.W. Lam:** Conceptualization, Formal analysis, Funding acquisition, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

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