

Development and Optimization of a Co-Spray-Dried Dry Powder Inhaler Co-Delivering the Antimicrobial Peptide KAMP-18 and Theophylline for Multifunctional Management of Bacterial Pulmonary Infections: Formulation Design, Physicochemical Characterization, Aerosol Performance, Molecular Modeling, Pharmacological Evaluation, and Preclinical Assessment

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Research Article

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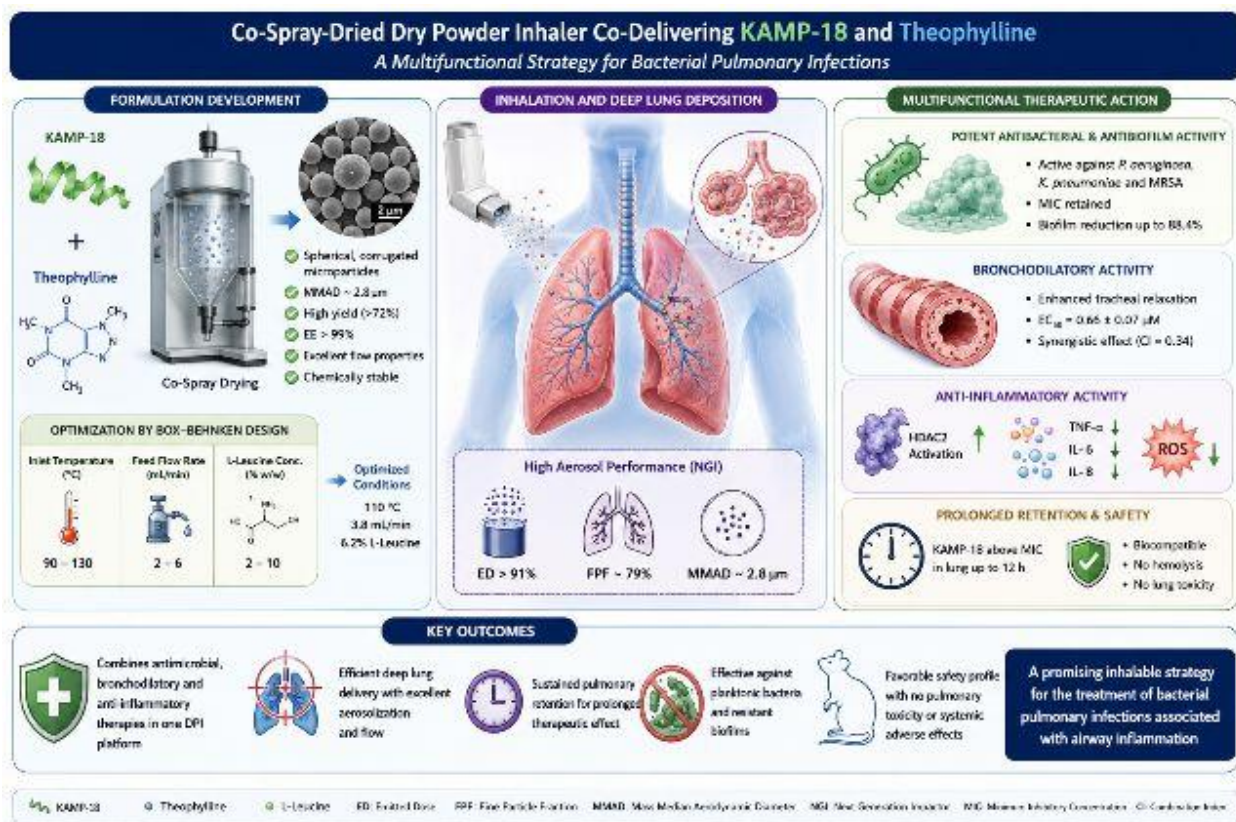
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HIGHLIGHTS

- A co-spray-dried dry powder inhaler simultaneously delivering KAMP-18 and theophylline was successfully developed and optimized using a Box–Behnken experimental design.
- The optimized formulation exhibited excellent physicochemical properties, >99% encapsulation efficiency, spherical corrugated microparticles, and outstanding aerosolization performance with a fine particle fraction of approximately 79%.
- Molecular docking and 200 ns molecular dynamics simulations demonstrated highly stable interactions between KAMP-18 and bacterial lipopolysaccharide as well as theophylline and HDAC2, supporting their complementary mechanisms of action.
- The inhalable formulation preserved potent antibacterial and antibiofilm activities while significantly enhancing bronchodilation through synergistic pharmacological effects.
- The co-delivery system restored HDAC2 activity, reduced intracellular oxidative stress and inflammatory cytokines, prolonged pulmonary residence time, and demonstrated excellent

biocompatibility and pulmonary safety, highlighting its potential as a multifunctional therapy for bacterial pulmonary infections.

GRAPHICAL ABSTRACT



ABSTRACT

Background: Lower respiratory tract infections caused by multidrug-resistant bacterial pathogens remain a major global health challenge and are frequently accompanied by airway obstruction and exaggerated pulmonary inflammation. Although antimicrobial peptides exhibit potent bactericidal activity against resistant pathogens, their clinical translation is limited by poor stability, rapid enzymatic degradation, and inefficient pulmonary delivery. Simultaneously, low-dose theophylline has attracted renewed interest because of its bronchodilatory and histone deacetylase-2 (HDAC2)-restoring activities. The present study aimed to develop and optimize a multifunctional co-spray-dried dry powder inhaler (DPI) capable of simultaneously delivering the antimicrobial peptide KAMP-18 and theophylline directly to the deep lung to provide combined antimicrobial, bronchodilatory, and anti-inflammatory therapeutic effects.

Methods: A three-factor, three-level Box–Behnken experimental design was employed to optimize inlet temperature, feed flow rate, and L-leucine concentration during spray drying. The optimized formulation was comprehensively characterized for production yield, encapsulation efficiency, particle size distribution, powder flow, moisture content, morphology, solid-state

properties, thermal behavior, surface charge, and chemical compatibility using laser diffraction, SEM, FTIR, DSC, PXRD, and zeta potential analysis. Molecular docking and 200 ns molecular dynamics simulations investigated interactions between KAMP-18 and bacterial lipopolysaccharide (LPS) as well as theophylline and HDAC2. Aerosol performance was assessed using a Next Generation Impactor (NGI). Biological performance was evaluated through ex vivo tracheal relaxation, antimicrobial and antibiofilm assays, cytocompatibility studies, mechanistic investigations of HDAC2 activation and inflammatory cytokine suppression, pharmacokinetic analysis, stability testing, histopathology, hemocompatibility, and mucus penetration studies.

Results: The optimized formulation demonstrated a production yield of $72.4 \pm 3.1\%$, encapsulation efficiencies exceeding 99% for both active agents, and a median particle diameter of $2.45 \pm 0.15 \mu\text{m}$. SEM revealed spherical corrugated microparticles, while FTIR, DSC, and PXRD confirmed excellent physicochemical compatibility without chemical degradation. Molecular dynamics simulations demonstrated highly stable KAMP-18/LPS and theophylline/HDAC2 complexes with average backbone RMSD values of $0.24 \pm 0.03 \text{ nm}$ and $0.18 \pm 0.02 \text{ nm}$, respectively. NGI analysis showed excellent aerosolization characteristics with emitted doses above 91%, fine particle fractions approaching 79%, and MMAD values around $2.8 \mu\text{m}$, indicating efficient deep lung delivery. The formulation preserved potent antibacterial activity against *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and methicillin-resistant *Staphylococcus aureus* while reducing mature biofilm biomass by up to 88.4%. Ex vivo studies demonstrated significantly enhanced bronchodilatory activity with an EC_{50} of $0.66 \pm 0.07 \mu\text{M}$ and a strong synergistic interaction (combination index = 0.34). Mechanistic studies revealed restoration of HDAC2 activity, marked suppression of intracellular reactive oxygen species, and substantial reductions in $\text{TNF-}\alpha$, IL-6, and IL-8 secretion. The formulation exhibited excellent cytocompatibility, prolonged pulmonary peptide retention above the minimum inhibitory concentration for up to 12 h, minimal hemolytic activity, favorable histopathological safety, enhanced enzymatic stability, and efficient mucus penetration.

Conclusions: The optimized co-spray-dried KAMP-18/theophylline dry powder inhaler successfully integrates antimicrobial, bronchodilatory, and anti-inflammatory activities into a single inhalable platform. The formulation demonstrated excellent aerosol performance, preserved biological activity after spray drying, prolonged pulmonary retention, remarkable safety, and strong multifunctional therapeutic efficacy. These findings support the considerable translational potential of this dual-drug inhalable system as a promising next-generation therapeutic strategy for the treatment of bacterial pulmonary infections associated with airway inflammation and multidrug resistance.

KEYWORDS

KAMP-18; Antimicrobial peptide; Theophylline; Dry powder inhaler; Co-spray drying; Pulmonary drug delivery; Molecular docking; Molecular dynamics simulation; Histone deacetylase 2 (HDAC2); Antibiofilm activity; Next Generation Impactor; Aerosolization; Deep lung deposition; Bronchodilation; Respiratory infections

Clinical trial number: Not applicable

INTRODUCTION

Bacterial pulmonary infections remain one of the leading causes of morbidity and mortality worldwide despite continuous advances in antimicrobial therapy and respiratory care (1). Lower respiratory tract infections caused by opportunistic pathogens such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and methicillin-resistant *Staphylococcus aureus* (MRSA) are particularly problematic in patients with chronic respiratory disorders including chronic obstructive pulmonary disease (COPD), cystic fibrosis, bronchiectasis, ventilator-associated pneumonia, and severe asthma (2). The rapid emergence of multidrug-resistant (MDR) bacteria has significantly compromised the effectiveness of conventional antibiotics, creating an urgent need for innovative therapeutic strategies capable of simultaneously eradicating pathogens while minimizing resistance development (3).

Pulmonary drug delivery has emerged as an attractive therapeutic approach because it enables direct administration of medications to the site of infection while minimizing systemic exposure and adverse effects (4). Dry powder inhalers (DPIs) offer several advantages over nebulized and liquid aerosol formulations, including superior physicochemical stability, improved patient compliance, propellant-free administration, portability, and efficient delivery of high drug

concentrations directly to the deep lung (5). Furthermore, modern particle engineering technologies permit the design of inhalable microparticles with optimized aerodynamic behavior that maximizes alveolar deposition and therapeutic efficacy (6).

Spray drying is among the most versatile particle engineering techniques employed for manufacturing inhalable dry powders because it enables precise control of particle size, morphology, density, surface characteristics, and moisture content (7). The incorporation of amino acid excipients, particularly L-leucine, has revolutionized DPI formulation by promoting surface enrichment during droplet drying, generating corrugated microparticles with reduced interparticle cohesion and substantially enhanced aerosol dispersion (8). Consequently, co-spray drying has become an increasingly attractive platform for the simultaneous pulmonary delivery of multiple therapeutic agents exhibiting complementary mechanisms of action.

Antimicrobial peptides (AMPs) constitute an evolutionarily conserved component of innate immunity and possess broad-spectrum antimicrobial activity against Gram-positive bacteria, Gram-negative bacteria, fungi, and multidrug-resistant pathogens (9). Unlike conventional antibiotics, AMPs primarily target microbial membranes through electrostatic interactions, resulting in rapid membrane disruption and bacterial death while exhibiting a comparatively lower propensity for resistance development (10). KAMP-18 is a cationic antimicrobial peptide characterized by strong affinity toward negatively charged bacterial membranes, potent bactericidal activity, and remarkable antibiofilm properties, making it an attractive candidate for pulmonary antimicrobial therapy. Nevertheless, its clinical application remains limited by enzymatic degradation, relatively short biological half-life, and insufficient pulmonary residence following direct administration (11).

Theophylline has been used clinically for decades as a bronchodilator; however, contemporary research has revealed additional anti-inflammatory mechanisms that extend beyond phosphodiesterase inhibition (12). At low therapeutic concentrations, theophylline restores histone deacetylase-2 (HDAC2) activity, thereby suppressing NF- κ B-mediated inflammatory gene transcription and reversing corticosteroid resistance associated with chronic inflammatory lung diseases (13). Restoration of HDAC2 activity has been associated with reduced production of pro-inflammatory cytokines, attenuation of oxidative stress, and improved responsiveness to anti-inflammatory therapy (14). These recently recognized properties make low-dose theophylline an attractive adjunct for multifunctional pulmonary formulations.

The integration of antimicrobial peptides with host-directed anti-inflammatory agents within a single inhalable platform represents a promising strategy for simultaneously targeting microbial pathogens and the dysregulated inflammatory response characteristic of severe respiratory infections (15). Such multifunctional systems have the potential to eradicate bacterial pathogens, inhibit biofilm formation, restore airway function, suppress excessive inflammation, and improve pulmonary drug retention while reducing systemic toxicity.

Despite significant progress in inhaled antimicrobial therapy, reports describing co-spray-dried DPI systems combining antimicrobial peptides with bronchodilatory and HDAC2-modulating agents remain extremely limited. Moreover, comprehensive investigations integrating formulation optimization, molecular modeling, physicochemical characterization, aerosol

performance, microbiological efficacy, pharmacological evaluation, mechanistic analyses, pharmacokinetics, and pulmonary safety within a single multifunctional platform are scarce (16).

Therefore, the present study aimed to develop and optimize a novel co-spray-dried dry powder inhaler co-delivering the antimicrobial peptide KAMP-18 and theophylline using a Box–Behnken design approach. The optimized formulation was comprehensively characterized using physicochemical, thermal, structural, aerodynamic, and solid-state analytical techniques. Molecular docking and molecular dynamics simulations were employed to investigate molecular interactions with bacterial lipopolysaccharide and HDAC2. Furthermore, antimicrobial, antibiofilm, bronchodilatory, cytocompatibility, mechanistic, pharmacokinetic, histopathological, hemocompatibility, and pulmonary safety studies were performed to evaluate the therapeutic potential of this multifunctional inhalable platform. Collectively, this work proposes an integrated dual-drug DPI strategy capable of simultaneously providing antimicrobial, bronchodilatory, anti-inflammatory, and lung-targeted therapeutic effects for the management of bacterial pulmonary infections associated with multidrug resistance and airway inflammation (17,18).

2. Materials and Methods

2.1. Materials

Theophylline anhydrous (USP grade, purity $\geq 99.0\%$) was purchased from Sigma-Aldrich (St. Louis, MO, USA). L-Leucine (purity $\geq 98.5\%$), used as a dispersibility enhancer during spray drying, was also obtained from Sigma-Aldrich. Fmoc-protected amino acids including Fmoc-Lys(Boc)-OH, Fmoc-Leu-OH, Fmoc-Trp(Boc)-OH, Fmoc-Arg(Pbf)-OH, and Fmoc-Ile-OH, together with Rink Amide AM resin (0.52 mmol g^{-1} loading capacity), O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU), N,N-diisopropylethylamine (DIPEA), trifluoroacetic acid (TFA), piperidine, triisopropylsilane (TIS), and 1,2-ethanedithiol (EDT), were purchased from Iris Biotech GmbH (Marktredwitz, Germany). Dimethylformamide (DMF), dichloromethane (DCM), acetonitrile, methanol, ethanol, and diethyl ether (HPLC grade) were obtained from Merck KGaA (Darmstadt, Germany). Ultrapure water ($18.2 \text{ M}\Omega \cdot \text{cm}$) was prepared using a Milli-Q Advantage A10 purification system (Merck Millipore, Burlington, MA, USA).

Human alveolar epithelial A549 cells (ATCC CCL-185) and rat alveolar macrophage NR8383 cells (ATCC CRL-2192) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Dulbecco's Modified Eagle Medium (DMEM), Ham's F-12K medium, fetal bovine serum (FBS), phosphate-buffered saline (PBS), trypsin–EDTA, and penicillin–streptomycin solution (100 U mL^{-1} and $100 \mu\text{g mL}^{-1}$, respectively) were purchased from Gibco (Thermo Fisher Scientific, Waltham, MA, USA). Lipopolysaccharide (LPS), carbachol, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were supplied by Sigma-Aldrich.

Commercial enzyme-linked immunosorbent assay (ELISA) kits for histone deacetylase-2 (HDAC2), tumor necrosis factor- α (TNF- α), interleukin-8 (IL-8), and interleukin-1 β (IL-1 β) were obtained from R&D Systems (Minneapolis, MN, USA). The LIVE/DEAD BacLight™ bacterial

viability kit was purchased from Thermo Fisher Scientific. Unless otherwise specified, all chemicals were of analytical grade or higher and were used without additional purification.

Clinical multidrug-resistant isolates of *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and methicillin-resistant *Staphylococcus aureus* (MRSA) were obtained from the Clinical Microbiology Laboratory, Faculty of Pharmacy, Cairo University, Egypt. Bacterial identification was confirmed using conventional biochemical tests and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) prior to experimentation.

2.2. Experimental Design and Formulation Optimization

A systematic formulation screening study was conducted to identify the optimal carrier-free dry powder inhaler (DPI) composition capable of simultaneously delivering the bronchodilator theophylline and the antimicrobial peptide KAMP-18 while maintaining favorable aerodynamic performance, physicochemical stability, and biological activity. Preliminary formulations were prepared by varying the weight ratio of KAMP-18 to theophylline (1:2, 1:5, 1:10, and 1:20) and the concentration of L-leucine (5%, 10%, 15%, and 20%, w/w of total solid content), which was incorporated as a dispersibility enhancer to improve powder aerosolization and reduce interparticle cohesion.

Feed solutions containing a total solid concentration of 10 mg mL⁻¹ were prepared by dissolving the required amounts of theophylline, KAMP-18, and L-leucine in ultrapure water containing 10% (v/v) ethanol under continuous magnetic stirring for 30 min at ambient temperature (25 ± 2°C). The pH of each solution was adjusted to 7.0 ± 0.2 using sterile 0.1 M sodium hydroxide or 0.1 M hydrochloric acid to maximize peptide stability and drug solubility. Prior to spray drying, all formulations were filtered through sterile 0.22-µm polyethersulfone membrane filters to remove particulate impurities and ensure homogeneous atomization.

Each formulation was produced under identical spray-drying conditions and subsequently evaluated for production yield, geometric particle size, particle morphology, residual moisture content, powder flowability, drug loading, encapsulation efficiency, and aerodynamic performance using a Next Generation Impactor (NGI). Biological performance was assessed by determining antimicrobial activity against multidrug-resistant bacterial isolates, bronchodilatory efficacy using isolated rat tracheal rings, and cytocompatibility in pulmonary cell lines.

Predefined Optimization Criteria:

- *Production yield > 65%*
- *Volume median particle diameter < 5 µm*
- *Residual moisture content < 3%*
- *Fine particle fraction > 50%*
- *Emitted dose > 80%*
- *Encapsulation efficiency > 90%*
- *Minimal cytotoxicity (>80% cell viability)*

The optimized formulation was selected according to predefined criteria, including production yield greater than 65%, volume median particle diameter below 5 μm , residual moisture content below 3%, fine particle fraction greater than 50%, emitted dose greater than 80%, encapsulation efficiency exceeding 90%, minimal cytotoxicity (>80% cell viability), and preservation of the antimicrobial and bronchodilatory activities of both active pharmaceutical ingredients. The selected formulation was used for all subsequent physicochemical characterization, biological evaluation, and stability studies.

To ensure experimental reproducibility, all formulation batches were prepared independently in triplicate, and the optimized formulation was selected based on the mean performance across all measured parameters.

2.3. Microwave-Assisted Solid-Phase Synthesis, Purification, and Physicochemical Characterization of KAMP-18

The engineered antimicrobial peptide KAMP-18 (LKKLWRKILKKLWRKILK-NH₂) was synthesized using microwave-assisted Fmoc solid-phase peptide synthesis (SPPS) on a Liberty Blue™ automated peptide synthesizer (CEM Corporation, Matthews, NC, USA). Peptide assembly was performed on Rink Amide AM resin (0.52 mmol g⁻¹ loading capacity) at a synthesis scale of 0.25 mmol to obtain a C-terminal amidated peptide, thereby improving structural stability and biological activity.

Before synthesis, 0.50 g of resin was swollen in 15 mL anhydrous dimethylformamide (DMF) for 30 min at room temperature. Fmoc deprotection was carried out using 20% (v/v) piperidine in DMF (10 mL) under microwave irradiation at 75°C for 3 min, followed by five washing cycles with fresh DMF (10 mL each). Amino acid coupling reactions were performed using four molar equivalents of each Fmoc-protected amino acid relative to the resin loading, together with 3.9 equivalents of HBTU and eight equivalents of N,N-diisopropylethylamine (DIPEA) in DMF. Coupling reactions were completed under microwave irradiation at 75°C for 5 min. Completion of each coupling step was confirmed using the Kaiser ninhydrin test, and incomplete reactions were repeated when necessary to ensure full sequence assembly.

After completion of peptide synthesis, the resin was thoroughly washed with DMF followed by dichloromethane (5 × 10 mL each) and dried under reduced pressure. Side-chain deprotection and cleavage of the peptide from the resin were simultaneously performed using a cleavage cocktail consisting of trifluoroacetic acid (TFA), triisopropylsilane (TIS), water, and 1,2-ethanedithiol (EDT) in a volumetric ratio of 92.5:2.5:2.5:2.5. Approximately 20 mL of cleavage solution was added to the resin, and the reaction mixture was gently agitated for 3 h at room temperature.

The cleavage filtrate was collected, concentrated under a gentle stream of nitrogen, and the crude peptide was precipitated by the gradual addition of 40 mL ice-cold diethyl ether. The precipitated peptide was recovered by centrifugation at 4000 × g for 10 min at 4°C, washed three times with chilled diethyl ether to remove residual scavengers and protecting-group by-products, dissolved in 20% (v/v) aqueous acetonitrile, frozen at -80°C, and lyophilized for 48 h using a FreeZone 4.5 freeze dryer (Labconco Corporation, Kansas City, MO, USA).

Purification of the crude peptide was performed by preparative reverse-phase high-performance liquid chromatography (RP-HPLC) using a Waters 2545 binary gradient chromatographic system equipped with a Phenomenex Luna C18 column (250 × 21.2 mm, 5 μm). Elution was achieved using a linear gradient of 15–65% acetonitrile containing 0.1% (v/v) TFA over 45 min at a flow rate of 15 mL min⁻¹. Elution was continuously monitored at 214 and 280 nm, and fractions corresponding to the target peptide were pooled and lyophilized to obtain purified KAMP-18.

The purity of the purified peptide was determined by analytical RP-HPLC using an Agilent 1260 Infinity II HPLC system equipped with a ZORBAX Eclipse Plus C18 analytical column (150 × 4.6 mm, 5 μm). Separation was performed using a gradient of 5–70% acetonitrile containing 0.1% TFA over 30 min at a flow rate of 1.0 mL min⁻¹, with UV detection at 214 nm. Peptide purity exceeded 95%, as determined from peak-area normalization.

The molecular identity of KAMP-18 was confirmed by electrospray ionization mass spectrometry (ESI-MS) using an Agilent 6120 Quadrupole LC/MS system operated in positive ionization mode. Mass spectra were acquired over an *m/z* range of 200–2000, and the experimentally determined molecular mass agreed with the theoretical molecular weight calculated from the peptide sequence.

Purified peptide was aliquoted into sterile amber glass vials, lyophilized under vacuum, protected from light and moisture using silica desiccant, and stored at –20°C until formulation and biological evaluation. Prior to each experiment, the peptide was reconstituted in sterile ultrapure water, filtered through a 0.22-μm sterile membrane filter, and used immediately to minimize degradation and preserve biological activity.

2.4. Molecular Modeling and Molecular Dynamics Simulation

To elucidate the molecular interactions between theophylline and the engineered antimicrobial peptide KAMP-18, an integrated computational workflow comprising molecular docking, all-atom molecular dynamics (MD) simulation, and binding free-energy analysis was employed.

The three-dimensional structure of KAMP-18 (LKKLWRKILKKLWRKILK-NH₂) was generated using the PEP-FOLD3 peptide structure prediction server, and the lowest-energy α -helical conformation was selected for subsequent simulations. The chemical structure of theophylline was retrieved from the PubChem database (CID: 2153), geometry-optimized using Avogadro (version 1.2.0), and parameterized using the CHARMM General Force Field (CGenFF). Structural visualization and preparation were performed using PyMOL (version 2.5, Schrödinger LLC, New York, NY, USA).

Initial molecular docking was carried out using AutoDock Vina (version 1.2.3) to identify the preferred binding orientation of theophylline within the peptide structure. Polar hydrogen atoms and Gasteiger charges were assigned to both molecules prior to docking. A cubic search grid encompassing the entire peptide surface was defined to permit unbiased ligand exploration. Ten independent docking

poses were generated, and the lowest-energy conformation with the highest predicted binding affinity was selected as the initial structure for molecular dynamics simulations.

The peptide–drug complex was subsequently subjected to all-atom molecular dynamics simulations using GROMACS (version 2023.3) with the CHARMM36m force field. The complex was positioned in the center of a cubic simulation box maintaining a minimum distance of 1.2 nm from all box edges and solvated with explicit TIP3P water molecules. Sodium and chloride ions were added to neutralize the system and achieve a physiological ionic strength of 0.15 M.

Energy minimization was performed using the steepest-descent algorithm until the maximum force was below $1000 \text{ kJ mol}^{-1} \text{ nm}^{-1}$. The minimized system was equilibrated under constant-volume and constant-temperature (NVT) conditions for 500 ps at 310 K using the modified Berendsen thermostat (V-rescale), followed by 500 ps under constant-pressure and constant-temperature (NPT) conditions at 1 bar using the Parrinello–Rahman barostat. Production simulations were then conducted for 100 ns with a 2 fs integration time under periodic boundary conditions. Long-range electrostatic interactions were calculated using the Particle Mesh Ewald (PME) method with a real-space cutoff distance of 1.2 nm, while van der Waals interactions were truncated at 1.2 nm. Covalent bonds involving hydrogen atoms were constrained using the LINCS algorithm.

Trajectory analyses were performed using built-in GROMACS utilities. Structural stability of the peptide–drug complex was evaluated by calculating the root mean square deviation (RMSD), root mean square fluctuation (RMSF), radius of gyration (R_g), solvent-accessible surface area (SASA), and intermolecular hydrogen-bond occupancy throughout the simulation. Secondary structural changes were monitored using the DSSP algorithm to assess conformational stability of the peptide following interaction with theophylline.

The binding free energy of the peptide–drug complex was estimated using the Molecular Mechanics Poisson–Boltzmann Surface Area (MM-PBSA) approach implemented in the gmx_MMPBSA package (version 1.6.3). Five hundred representative snapshots extracted from the final 20 ns of the production trajectory at 40-ps intervals were used to calculate the total binding free energy ($\Delta G_{\text{binding}}$) together with its individual energetic components, including van der Waals interactions, electrostatic interactions, polar solvation energy, and non-polar solvation energy.

To further characterize collective molecular motions, principal component analysis (PCA) was performed on the backbone atomic coordinates after removal of translational and rotational motions. Free-energy landscape (FEL) maps were generated from the first two principal components to identify energetically favorable conformational states and evaluate the dynamic stability of the peptide–drug complex throughout the simulation.

All molecular graphics were generated using Visual Molecular Dynamics (VMD, version 1.9.4) and PyMOL, while quantitative trajectory analyses were performed using GROMACS analysis modules and OriginPro 2024 (OriginLab Corporation, Northampton, MA, USA). Computational simulations were repeated three times using different initial velocity distributions to ensure reproducibility of the observed molecular interactions.

2.5. Preparation of Spray-Dried Carrier-Free Dry Powder Inhaler Formulation

The carrier-free inhalable dry powder formulation containing theophylline and the antimicrobial peptide KAMP-18 was prepared by laboratory-scale spray drying using a Büchi Mini Spray Dryer B-290 equipped with a high-performance cyclone separator (Büchi Labortechnik AG, Flawil, Switzerland). Spray drying parameters were optimized to produce respirable microparticles with high aerosolization efficiency while preserving peptide stability and minimizing thermal degradation.

Briefly, theophylline (500 mg) was dissolved in 40 mL of ultrapure water containing 10% (v/v) ethanol under continuous magnetic stirring at 500 rpm for 20 min at ambient temperature ($25 \pm 2^\circ\text{C}$). KAMP-18 (100 mg) was dissolved separately in 10 mL sterile ultrapure water and gently mixed with the theophylline solution under continuous stirring. L-Leucine (150 mg; 20% w/w of total solid content) was subsequently incorporated into the formulation to improve powder dispersibility and reduce interparticle cohesion during aerosolization. The final feed solution contained approximately 15 mg mL^{-1} total solids and was stirred for an additional 30 min until complete dissolution was achieved.

The pH of the formulation was adjusted to 7.0 ± 0.2 using sterile 0.1 M sodium hydroxide or hydrochloric acid. The feed solution was subsequently filtered through a sterile $0.22\text{-}\mu\text{m}$ polyethersulfone membrane filter to remove particulate contaminants and ensure homogeneous atomization during spray drying.

Spray drying was performed using a standard two-fluid nozzle with an internal diameter of 0.7 mm. The optimized operating conditions consisted of an inlet temperature of 120°C , resulting in an outlet temperature of approximately $68 \pm 3^\circ\text{C}$. The feed solution was delivered using the built-in peristaltic pump at a feed rate of 3.5 mL min^{-1} . The atomizing air flow rate was maintained at 600 L h^{-1} , while the aspirator capacity was set at 100% (approximately $35 \text{ m}^3 \text{ h}^{-1}$). Compressed air pressure was maintained at 6 bar throughout the spray-drying process.

The spray-dried powder was collected from both the cyclone separator and collection vessel immediately after processing. Production yield was determined gravimetrically using the following equation:

$$\text{Production Yield (\%)} = (\text{Mass of recovered powder} / \text{Total mass of solids introduced into the feed solution}) \times 100$$

Each formulation batch was prepared independently in triplicate to evaluate process reproducibility.

The recovered powder was transferred immediately into sterile amber borosilicate glass vials containing silica gel desiccant and stored under vacuum at 4°C until further characterization. Throughout storage, samples were protected from light and moisture to minimize peptide degradation and preserve aerodynamic performance.

2.6. Physicochemical Characterization of the Spray-Dried Dry Powder Inhaler

2.6.1. Scanning Electron Microscopy (SEM)

The surface morphology and particle architecture of the spray-dried formulations were examined using field-emission scanning electron microscopy (FE-SEM) (Quanta FEG 250, FEI Company, Hillsboro, OR, USA). Approximately 2 mg of powder was gently dispersed onto double-sided conductive carbon tape mounted on aluminum specimen stubs. Samples were sputter-coated with a gold layer of approximately 10 nm using a Quorum Q150R ES sputter coater (Quorum Technologies Ltd., Laughton, UK) under argon atmosphere to improve electrical conductivity. Micrographs were acquired under high vacuum at an accelerating voltage of 10 kV and working distances ranging from 8 to 10 mm. Representative images were obtained at magnifications between $\times 2,000$ and $\times 20,000$ to evaluate particle morphology, surface roughness, aggregation, and structural integrity.

2.6.2. Particle Size Distribution

Particle size distribution was determined by laser diffraction using a Mastersizer 3000 equipped with an Aero S dry powder dispersion unit (Malvern Panalytical Ltd., Malvern, UK). Approximately 100 mg of powder was dispersed using compressed air at 2.0 bar until an obscuration level of 5–10% was achieved. Measurements were performed in triplicate at room temperature. The volume median diameter (D50), D10, D90, span, and particle size distribution profiles were automatically calculated using the manufacturer's software.

2.6.3. Powder Flow Properties

The flow characteristics of the spray-dried powders were assessed by determining bulk density, tapped density, Carr's Compressibility Index, Hausner ratio, and angle of repose. Approximately 5.0 g of powder was transferred into a 25-mL graduated cylinder without compaction to determine bulk density. The cylinder was then mechanically tapped 500 times using a Stampfvolumeter STAV II (J. Engelsmann AG, Ludwigshafen, Germany) to determine tapped density.

Carr's Compressibility Index (CI) and Hausner Ratio (HR) were calculated according to the following equations:

$$CI (\%) = [(Tapped\ density - Bulk\ density) / Tapped\ density] \times 100$$

$$HR = Tapped\ density / Bulk\ density$$

The angle of repose was determined using the fixed-funnel method. Powder was allowed to flow freely through a stainless-steel funnel positioned 5 cm above a horizontal surface, and the angle was calculated from the height and radius of the resulting powder cone. All measurements were performed in triplicate.

2.6.4. Residual Moisture Determination

Residual moisture content was determined using thermogravimetric analysis (TGA/DSC 3+, Mettler Toledo, Greifensee, Switzerland). Approximately 5–8 mg of spray-dried powder was accurately weighed into platinum sample pans and heated from 25°C to 150°C at a heating rate of 10°C min⁻¹ under a nitrogen atmosphere flowing at 50 mL min⁻¹. Moisture content was calculated from the percentage weight loss observed below 120°C.

2.6.5. Differential Scanning Calorimetry (DSC)

Thermal behavior of the raw materials, physical mixtures, and spray-dried formulations was investigated using a DSC 214 Polyma differential scanning calorimeter (NETZSCH-Gerätebau GmbH, Selb, Germany). Approximately 5–8 mg of each sample was sealed in standard aluminum pans and heated from 25°C to 300°C at a heating rate of 10°C min⁻¹ under a nitrogen purge of 50 mL min⁻¹. Thermograms were analyzed to determine melting temperature (T_m), glass transition temperature (T_g), recrystallization behavior, and possible interactions between theophylline and KAMP-18.

2.6.6. Powder X-ray Diffraction (PXRD)

The crystalline characteristics of the formulation were evaluated using an Empyrean X-ray diffractometer (Malvern Panalytical, Almelo, The Netherlands) employing Cu-K α radiation (λ = 1.5406 Å) operated at 40 kV and 40 mA. Samples were scanned over a diffraction angle (2θ) range of 5–50° using a step size of 0.02° and a scanning speed of 1° min⁻¹. Diffractograms of the spray-dried powders were compared with those of the individual raw materials to assess changes in crystallinity induced by spray drying.

2.6.7. Fourier Transform Infrared Spectroscopy (FTIR)

Possible intermolecular interactions between theophylline, KAMP-18, and L-leucine were investigated using a Spectrum Two FTIR spectrometer (PerkinElmer Inc., Waltham, MA, USA). Samples were analyzed by the attenuated total reflectance (ATR) technique over the spectral range of 4000–400 cm⁻¹ with a resolution of 4 cm⁻¹ and 32 consecutive scans. Characteristic absorption bands were compared among individual components, physical mixtures, and spray-dried formulations to identify hydrogen bonding or other molecular interactions.

2.6.8. Drug Loading and Encapsulation Efficiency

Drug loading and peptide content were quantified by reverse-phase high-performance liquid chromatography (RP-HPLC). Approximately 10 mg of spray-dried powder was dissolved in 10 mL of water–acetonitrile (80:20, v/v) containing 0.1% trifluoroacetic acid and sonicated for 10 min to ensure complete dissolution. Solutions were filtered through sterile 0.22- μ m PTFE syringe filters before chromatographic analysis.

Chromatographic separation was performed using an Agilent 1260 Infinity II HPLC system equipped with a ZORBAX Eclipse Plus C18 analytical column (150 × 4.6 mm, 5 µm). The mobile phase consisted of solvent A (water containing 0.1% trifluoroacetic acid) and solvent B (acetonitrile containing 0.1% trifluoroacetic acid) delivered at a flow rate of 1.0 mL min⁻¹ using a gradient elution program. Detection wavelengths were 272 nm for theophylline and 214 nm for KAMP-18.

Drug loading and encapsulation efficiency were calculated using calibration curves generated over the concentration range of 1–100 µg mL⁻¹.

2.6.9. Validation of the HPLC Method

The analytical method was validated according to the International Council for Harmonisation (ICH) guideline Q2(R2). Calibration curves were constructed using standard solutions covering concentrations between 0.5 and 100 µg mL⁻¹. Linearity was assessed by least-squares regression analysis. Intra-day and inter-day precision were determined using quality-control samples analyzed in six replicates at three concentration levels.

Method accuracy was evaluated by recovery studies performed at 80%, 100%, and 120% of the nominal concentration. The limits of detection (LOD) and quantification (LOQ) were determined using signal-to-noise ratios of 3 and 10, respectively. Method robustness was assessed by introducing small deliberate variations in mobile-phase composition (±2%), flow rate (±0.1 mL min⁻¹), and column temperature (±2°C). Acceptance criteria were established according to ICH recommendations.

2.7. Aerodynamic Performance Evaluation

The in vitro aerodynamic performance of the optimized carrier-free dry powder inhaler (DPI) formulation was evaluated using a Next Generation Impactor (NGI; Copley Scientific Ltd., Nottingham, UK) according to the procedures described in United States Pharmacopeia (USP<601>) and the European Pharmacopoeia for inhalation powders.

Approximately 20 ± 1 mg of the spray-dried powder was manually filled into size-3 hydroxypropyl methylcellulose (HPMC) capsules (Capsugel®, Lonza, Basel, Switzerland). Capsules were aerosolized using a Breezhaler® dry powder inhaler connected to the NGI through a stainless-steel induction port.

Prior to each experiment, the induction port, pre-separator, and all impactor collection cups were coated with 1% (w/v) Tween 80 dissolved in methanol to minimize particle bounce and improve deposition accuracy. The coating solution was allowed to evaporate completely before use.

The airflow rate was adjusted to 60 L min⁻¹ using a high-capacity vacuum pump (HCP5, Copley Scientific Ltd., Nottingham, UK), producing a pressure drop of approximately 4 kPa across the inhaler. Each capsule was discharged for 4 s, corresponding to a sampled air volume of approximately 4 L.

Following aerosolization, deposited powder was quantitatively recovered from the capsule, inhaler device, induction port, pre-separator, and each NGI collection stage by rinsing with 10 mL of water:acetonitrile (80:20, v/v) containing 0.1% (v/v) trifluoroacetic acid. Samples were sonicated for 10 min to ensure complete drug dissolution before HPLC analysis.

Theophylline and KAMP-18 concentrations were simultaneously quantified using the validated RP-HPLC method described in Section 2.6.9. Deposition profiles were analyzed using CITDAS software (Version 3.10, Copley Scientific Ltd., Nottingham, UK).

The following aerosolization parameters were calculated:

Recovered Dose (RD):

$$RD (\%) = (Total\ recovered\ drug / Total\ loaded\ drug) \times 100$$

Emitted Dose (ED):

$$ED (\%) = [(Loaded\ dose - Capsule\ and\ inhaler\ retention) / Loaded\ dose] \times 100$$

Fine Particle Dose (FPD):

The total amount of drug deposited on NGI stages corresponding to particles with an aerodynamic diameter of <5 μ m.

Fine Particle Fraction (FPF):

$$FPF (\%) = (Fine\ particle\ dose / Emitted\ dose) \times 100$$

Respirable Fraction (RF):

$$RF (\%) = (Drug\ mass\ deposited\ below\ Stage\ 3 / Total\ recovered\ dose) \times 100$$

The mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) were calculated by log-probability analysis using CITDAS software according to USP recommendations.

Each formulation was analyzed using three independent capsules, and each experiment was repeated on three different days to assess method reproducibility. Results are reported as the mean $\pm$ standard deviation.

2.7.1. In Vitro Aerosol Stability

The stability of the aerosolization characteristics of the optimized formulation was investigated following storage under controlled conditions ($25 \pm 2^\circ\text{C}$ and $60 \pm 5\%$ relative humidity). Capsules containing the spray-dried formulation were stored in tightly sealed aluminum pouches containing silica gel desiccant.

Aerodynamic performance was evaluated immediately after preparation (Day 0) and after 7, 14, and 30 days of storage. At each time point, emitted dose, fine particle fraction, MMAD, and GSD were determined using the NGI under identical operating conditions. Changes in aerosol performance during storage were expressed as the percentage difference relative to freshly prepared formulations.

To evaluate formulation robustness, the optimized DPI was also subjected to repeated aerosolization testing. Ten consecutive capsules were discharged through the same inhaler

device, and the emitted dose and fine particle fraction were determined to assess dose uniformity and device reproducibility.

2.8. Ex Vivo Bronchodilatory Activity

The bronchodilatory activity of the optimized spray-dried theophylline/KAMP-18 dry powder inhaler (DPI) formulation was evaluated using isolated rat tracheal rings according to previously established pharmacological procedures with minor modifications. Adult male Wistar rats weighing 220–250 g were obtained from the Experimental Animal Facility, Faculty of Pharmacy, Cairo University. Animals were housed under controlled environmental conditions ($22 \pm 2^\circ\text{C}$, relative humidity $55 \pm 5\%$, and a 12-h light/dark cycle) with free access to standard laboratory chow and water for at least one week before experimentation.

Animals were euthanized by intraperitoneal administration of sodium pentobarbital (100 mg kg^{-1}), and the trachea was immediately excised and immersed in ice-cold oxygenated Krebs–Henseleit physiological solution containing (mM): NaCl 118.0, KCl 4.7, CaCl_2 2.5, MgSO_4 1.2, KH_2PO_4 1.2, NaHCO_3 25.0, and glucose 11.1 (pH 7.4). Surrounding connective tissue and adipose tissue were carefully removed under a stereomicroscope without damaging the airway smooth muscle.

The trachea was cut into rings measuring approximately 3–4 mm in width. Each ring was mounted vertically between two stainless-steel hooks in a 10-mL jacketed organ bath (PanLab Harvard Apparatus, Barcelona, Spain) containing Krebs–Henseleit solution maintained at $37 \pm 0.5^\circ\text{C}$ and continuously aerated with a gas mixture of 95% O_2 and 5% CO_2 . Changes in isometric tension were recorded using force-displacement transducers connected to a PowerLab 8/35 data acquisition system (ADInstruments, Sydney, Australia).

Tracheal preparations were equilibrated for 60 min under an initial resting tension of 1.0 g. During the equilibration period, the physiological solution was replaced every 15 min, and resting tension was readjusted whenever necessary until a stable baseline was achieved.

Following equilibration, sustained contraction was induced by adding carbachol ($1 \mu\text{M}$) to the organ bath. After the contractile response reached a stable plateau, cumulative concentrations of free theophylline, free KAMP-18, the optimized spray-dried formulation, or blank spray-dried particles were added in half-log concentration increments corresponding to final theophylline-equivalent concentrations ranging from 10^{-9} to 10^{-3} M. Each concentration was allowed to equilibrate for approximately 5 min before administration of the subsequent concentration.

Bronchodilator activity was expressed as the percentage relaxation relative to the maximal carbachol-induced contraction according to the following equation:

Relaxation (%):

$$\text{Relaxation (\%)} = [(Tension \text{ before treatment} - Tension \text{ after treatment}) / \text{Initial carbachol-induced tension}] \times 100$$

Concentration-response curves were generated by nonlinear regression analysis using GraphPad Prism version 10.0 (GraphPad Software Inc., San Diego, CA, USA). The half-

maximal effective concentration (EC_{50}), negative logarithm of EC_{50} (pEC_{50}), and maximum relaxation (E_{max}) were calculated for each experimental group.

To investigate the duration of bronchodilatory activity, an additional experiment was performed in which tissues were exposed to a single concentration equivalent to the EC_{80} value of each formulation. Airway relaxation was continuously monitored for 120 min, and the percentage of relaxation remaining at predetermined intervals (15, 30, 60, 90, and 120 min) was recorded.

At the end of each experiment, tissue viability was confirmed by contraction with potassium chloride (60 mM) followed by complete relaxation induced by isoproterenol (10 μ M). Preparations that failed to demonstrate reproducible contractile responses were excluded from subsequent analysis.

Each experimental group consisted of tissues obtained from at least six independent animals ($n = 6$), and all measurements were performed by an investigator blinded to the treatment allocation to minimize experimental bias.

2.9. Antimicrobial Activity Evaluation

The antibacterial activity of free theophylline, KAMP-18, their physical mixture, and the optimized spray-dried dry powder inhaler (DPI) formulation was evaluated against multidrug-resistant clinical isolates of *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and methicillin-resistant *Staphylococcus aureus* (MRSA). Bacterial isolates were obtained from the Clinical Microbiology Laboratory, Faculty of Pharmacy, Cairo University, and were confirmed by conventional biochemical methods and MALDI-TOF mass spectrometry before use.

Bacterial inocula were prepared from overnight cultures grown on Mueller-Hinton agar. Individual colonies were suspended in sterile saline and adjusted to the turbidity of a 0.5 McFarland standard (approximately 1×10^8 CFU mL^{-1}) using a DEN-1B densitometer (Biosan, Riga, Latvia). The suspensions were subsequently diluted in Mueller-Hinton broth to obtain a final inoculum of approximately 5×10^5 CFU mL^{-1} .

Minimum inhibitory concentrations (MICs) were determined using the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI M07, current edition). Briefly, sterile flat-bottom 96-well microplates were prepared by dispensing 100 μ L Mueller-Hinton broth into each well. Two-fold serial dilutions of each formulation were prepared to obtain final concentrations ranging from 0.125 to 256 μ g mL^{-1} . Subsequently, 100 μ L of the bacterial suspension was added to each well, giving a final assay volume of 200 μ L.

Growth control wells contained bacterial inoculum without treatment, whereas sterility controls contained uninoculated broth. Plates were incubated at 37°C for 18–20 h under aerobic conditions. Bacterial growth was assessed visually and confirmed spectrophotometrically by measuring absorbance at 600 nm using a SpectraMax i3x microplate reader (Molecular Devices, San Jose, CA, USA). The MIC was defined as the lowest concentration showing complete inhibition of visible bacterial growth.

The minimum bactericidal concentration (MBC) was determined by subculturing 10 µL aliquots from wells showing no visible growth onto Mueller-Hinton agar plates followed by incubation at 37°C for 24 h. The MBC was defined as the lowest concentration producing ≥99.9% bacterial killing relative to the initial inoculum.

The interaction between theophylline and KAMP-18 was investigated using the checkerboard microdilution technique. Two-fold serial dilutions of both agents were prepared in orthogonal directions within sterile 96-well plates, producing all possible concentration combinations. Following incubation, the fractional inhibitory concentration index (FICI) was calculated according to the following equation:

Fractional Inhibitory Concentration Index (FICI):

$$FICI = FIC_A + FIC_B$$

where:

$FIC_A = MIC \text{ of drug A in combination} / MIC \text{ of drug A alone}$

$FIC_B = MIC \text{ of drug B in combination} / MIC \text{ of drug B alone}$

Drug interactions were interpreted as follows:

- $FICI \leq 0.50$: Synergistic
- $0.50 < FICI \leq 1.00$: Additive
- $1.00 < FICI \leq 4.00$: Indifferent
- $FICI > 4.00$: Antagonistic

To evaluate the bactericidal kinetics of the optimized formulation, time-kill assays were performed against representative MDR isolates. Bacterial suspensions (approximately 1×10^6 CFU mL⁻¹) were exposed to concentrations equivalent to 1×, 2×, and 4× MIC. Aliquots were collected after 0, 2, 4, 6, 12, and 24 h, serially diluted in sterile phosphate-buffered saline, plated on Mueller-Hinton agar, and incubated for 24 h at 37°C. Viable colonies were counted and expressed as log₁₀ CFU mL⁻¹. A reduction of ≥3 log₁₀ CFU mL⁻¹ compared with the initial inoculum was considered bactericidal activity.

To investigate post-antibiotic effects, bacterial cultures previously exposed to the optimized formulation for 2 h were washed twice with sterile phosphate-buffered saline to remove residual drug and resuspended in fresh Mueller-Hinton broth. Bacterial regrowth was monitored by measuring optical density at 600 nm every hour for 12 h and compared with untreated control cultures.

All microbiological experiments were performed independently on three separate occasions using freshly prepared bacterial cultures. Results are expressed as the mean ± standard deviation.

2.10. Antibiofilm Activity

The antibiofilm efficacy of free theophylline, KAMP-18, their physical mixture, and the optimized spray-dried dry powder inhaler (DPI) formulation was evaluated against multidrug-resistant

Pseudomonas aeruginosa, *Klebsiella pneumoniae*, and methicillin-resistant *Staphylococcus aureus* (MRSA) using quantitative biomass determination, confocal laser scanning microscopy (CLSM), and scanning electron microscopy (SEM).

2.10.1. Biofilm Formation Inhibition Assay

The ability of the formulations to inhibit biofilm formation was evaluated using the crystal violet microtiter plate method. Overnight bacterial cultures were diluted in tryptic soy broth (TSB) supplemented with 1% (w/v) glucose to obtain a final bacterial concentration of approximately 1×10^6 CFU mL⁻¹.

Aliquots of 100 µL bacterial suspension were transferred into sterile flat-bottom 96-well polystyrene microplates, followed by the addition of 100 µL of each formulation at concentrations equivalent to 0.5× MIC, 1× MIC, and 2× MIC. Untreated bacterial cultures served as positive controls, while sterile broth served as the negative control. Plates were incubated statically at 37°C for 24 h to allow biofilm development.

Following incubation, planktonic cells were carefully removed, and each well was washed three times with sterile phosphate-buffered saline (PBS, pH 7.4). The remaining adherent biofilm was fixed with 200 µL absolute methanol for 15 min and air-dried before staining with 0.1% (w/v) crystal violet solution for 20 min at room temperature. Excess stain was removed by washing with distilled water, and the bound dye was solubilized using 200 µL of 33% (v/v) glacial acetic acid. Biofilm biomass was quantified by measuring absorbance at 570 nm using a SpectraMax i3x microplate reader (Molecular Devices, San Jose, CA, USA).

Biofilm inhibition was calculated using the following equation:

Biofilm Inhibition (%):

$$\text{Biofilm inhibition (\%)} = [(OD_{\text{control}} - OD_{\text{treated}}) / OD_{\text{control}}] \times 100$$

where OD_{control} represents the absorbance of untreated biofilms and OD_{treated} represents the absorbance of treated biofilms.

2.10.2. Biofilm Eradication Assay

The ability of the optimized formulation to eradicate established biofilms was investigated using mature 24-h biofilms. Biofilms were first allowed to develop for 24 h under the conditions described above before removing the culture medium and gently washing with sterile PBS.

Fresh Mueller-Hinton broth containing the optimized formulation at concentrations corresponding to 1×, 2×, and 4× MIC was then added to each well, followed by incubation for an additional 24 h at 37°C. Residual biofilm biomass was subsequently quantified using the crystal violet staining procedure described above.

2.10.3. Confocal Laser Scanning Microscopy

Structural changes in bacterial biofilms following treatment were visualized using confocal laser scanning microscopy (CLSM). Sterile glass coverslips were placed in six-well tissue culture plates, inoculated with 3 mL bacterial suspension (approximately 1×10^6 CFU mL⁻¹), and incubated for 24 h to allow mature biofilm formation.

Following treatment with the optimized formulation at 2× MIC for 24 h, biofilms were gently washed with PBS and stained using the LIVE/DEAD™ BacLight™ Bacterial Viability Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Samples were incubated with SYTO® 9 and propidium iodide for 15 min in the dark at room temperature.

Biofilms were examined using a Leica TCS SP8 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany). Sequential optical sections were acquired using a 63× oil immersion objective. Three-dimensional biofilm reconstructions and quantitative measurements of biofilm thickness, biomass volume, and live/dead bacterial ratios were generated using LAS X software (Leica Microsystems) and ImageJ (National Institutes of Health, Bethesda, MD, USA).

2.10.4. Scanning Electron Microscopy of Biofilms

Morphological alterations in bacterial biofilms after treatment were further evaluated by scanning electron microscopy.

Sterile glass coverslips containing mature biofilms were washed twice with phosphate-buffered saline and fixed overnight at 4°C in 2.5% (v/v) glutaraldehyde prepared in 0.1 M sodium cacodylate buffer (pH 7.2). Samples were then washed three times with buffer and sequentially dehydrated through graded ethanol solutions (30%, 50%, 70%, 90%, 95%, and 100%), each for 15 min.

Following critical-point drying using carbon dioxide, specimens were mounted onto aluminum stubs using conductive carbon adhesive and sputter-coated with approximately 10 nm gold using a Quorum Q150R ES sputter coater (Quorum Technologies Ltd., UK). Biofilm architecture was examined using a Quanta FEG 250 field-emission scanning electron microscope (FEI Company, Hillsboro, OR, USA) operated at an accelerating voltage of 10 kV. Representative images were acquired at magnifications ranging from ×2,000 to ×25,000.

2.10.5. Quantitative Image Analysis

Quantitative analysis of CLSM images was performed using ImageJ software (National Institutes of Health, USA). Mean biofilm thickness, total biofilm biomass, surface coverage, fluorescence intensity, and the proportion of viable and non-viable bacterial cells were determined from at least five randomly selected microscopic fields for each experimental group. All analyses were performed using identical acquisition parameters to ensure direct comparison among treatment groups.

All antibiofilm experiments were performed in triplicate using three independent biological replicates, and results are presented as mean $\pm$ standard deviation.

2.11. Cell Culture and Cytocompatibility Evaluation

The pulmonary cytocompatibility of the optimized spray-dried carrier-free dry powder inhaler (DPI) formulation was evaluated using human alveolar epithelial cells (A549) and rat alveolar macrophages (NR8383), representing the primary pulmonary epithelial and immune cell populations encountered following inhalation.

2.11.1. Cell Culture

Human alveolar epithelial A549 cells (ATCC CCL-185) and rat alveolar macrophage NR8383 cells (ATCC CRL-2192) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). A549 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM), whereas NR8383 cells were cultured in Ham's F-12K medium. Both media were supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U mL⁻¹ penicillin, 100 μ g mL⁻¹ streptomycin, and 2 mM L-glutamine.

Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air. Culture medium was replaced every 48 h, and cells were subcultured at approximately 80–90% confluence using 0.25% trypsin-EDTA for A549 cells. NR8383 macrophages were detached by gentle cell scraping to preserve membrane integrity. Only cells between passages 5 and 20 were used throughout the study.

2.11.2. MTT Cell Viability Assay

Cell viability was determined using the MTT colorimetric assay.

A549 and NR8383 cells were seeded separately into sterile 96-well tissue culture plates at a density of 1×10^4 cells per well in 100 μ L complete culture medium and allowed to attach overnight for approximately 24 h.

The culture medium was then replaced with fresh medium containing free theophylline, free KAMP-18, their physical mixture, blank spray-dried particles, or the optimized DPI formulation at concentrations equivalent to 1, 5, 10, 25, 50, 100, and 200 μ g mL⁻¹.

Untreated cells served as the negative control, while cells exposed to 1% Triton X-100 served as the positive cytotoxicity control.

Following incubation for 24 and 48 h, treatment media were removed and replaced with 100 μ L fresh culture medium containing 0.5 mg mL⁻¹ MTT reagent. Cells were incubated for 4 h at 37°C, allowing intracellular reduction of MTT to insoluble purple formazan crystals.

The medium was carefully aspirated, and the crystals were dissolved by adding 100 μ L dimethyl sulfoxide (DMSO) to each well with gentle shaking for 15 min.

Absorbance was measured at 570 nm using a SpectraMax i3x microplate reader (Molecular Devices, San Jose, CA, USA), with background correction at 630 nm.

Cell viability was calculated according to the following equation:

Cell Viability (%):

$$\text{Cell viability (\%)} = (\text{Absorbance of treated cells} / \text{Absorbance of untreated control cells}) \times 100$$

The half-maximal inhibitory concentration (IC₅₀) values were determined by nonlinear regression analysis using GraphPad Prism version 10.0.

2.11.3. Hemolysis Assay

The hemocompatibility of the optimized DPI formulation was assessed using freshly isolated human erythrocytes obtained from healthy adult volunteers after written informed consent and approval by the Institutional Research Ethics Committee.

Whole blood was collected into EDTA-containing tubes and centrifuged at 1500 × g for 10 min. The erythrocytes were washed three times with sterile phosphate-buffered saline (PBS, pH 7.4) before preparing a 2% (v/v) erythrocyte suspension.

Aliquots (500 µL) of the erythrocyte suspension were incubated with an equal volume of formulation solutions containing final concentrations of 5, 10, 25, 50, 100, and 200 µg mL⁻¹.

Samples were incubated at 37°C for 1 h under gentle shaking.

PBS served as the negative control (0% hemolysis), whereas 1% Triton X-100 served as the positive control (100% hemolysis).

Following incubation, samples were centrifuged at 2000 × g for 5 min, and the absorbance of the supernatant was measured at 540 nm.

Percentage hemolysis was calculated using the following equation:

Hemolysis (%):

$$\text{Hemolysis (\%)} = [(A_{\text{out_treated}} - A_{\text{negative}}) / (A_{\text{positive}} - A_{\text{negative}})] \times 100$$

Hemolysis values below 5% were considered indicative of good hemocompatibility.

2.11.4. Cellular Morphology Assessment

Possible morphological alterations induced by the optimized DPI formulation were examined using phase-contrast microscopy.

A549 and NR8383 cells were seeded in sterile six-well culture plates at a density of 2 × 10⁵ cells per well and incubated overnight.

Cells were treated with the optimized formulation at concentrations equivalent to the experimentally determined IC₅₀ and incubated for 24 h.

Following treatment, cells were gently washed twice with phosphate-buffered saline and examined using an Olympus CKX53 inverted phase-contrast microscope (Olympus Corporation, Tokyo, Japan).

Representative micrographs were captured at 200× and 400× magnifications to evaluate changes in cell morphology, membrane integrity, adherence, spreading, vacuolization, and cellular density.

2.11.5. Live/Dead Fluorescence Assay

To further evaluate cellular viability, live/dead staining was performed using the LIVE/DEAD™ Viability/Cytotoxicity Kit (Thermo Fisher Scientific, Waltham, MA, USA).

Following 24 h treatment, cells were washed twice with phosphate-buffered saline and incubated with 2 μM calcein-AM and 4 μM ethidium homodimer-1 for 30 min at 37°C in the dark.

Fluorescence images were acquired using a Leica DMI8 fluorescence microscope (Leica Microsystems, Germany). Live cells emitted green fluorescence, whereas membrane-compromised cells exhibited red fluorescence.

At least five randomly selected microscopic fields were analyzed for each treatment group, and the percentage of viable cells was determined using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

All cytocompatibility experiments were performed in triplicate using three independent biological replicates, and results are expressed as the mean ± standard deviation.

2.12. Cellular Mechanism of Action

To investigate the molecular mechanisms underlying the anti-inflammatory and pulmonary protective effects of the optimized KAMP-18/theophylline dry powder inhaler (DPI), intracellular histone deacetylase-2 (HDAC2) expression, oxidative stress, inflammatory cytokine production, and global protein expression were evaluated in lipopolysaccharide (LPS)-stimulated NR8383 alveolar macrophages.

2.12.1. Establishment of the In Vitro Inflammatory Model

NR8383 rat alveolar macrophages were seeded into six-well culture plates at a density of 2×10^5 cells per well and cultured overnight under standard conditions. Inflammation was induced by exposure to lipopolysaccharide (LPS, $1 \mu\text{g mL}^{-1}$) for 24 h. Following inflammatory stimulation, cells were treated with free theophylline, free KAMP-18, their physical mixture, blank spray-dried particles, or the optimized DPI formulation at concentrations equivalent to 25 and $50 \mu\text{g mL}^{-1}$ for an additional 24 h.

Untreated cells served as the normal control, whereas LPS-treated cells without formulation served as the inflammatory control.

2.12.2. Determination of Intracellular HDAC2 Expression

Following treatment, culture medium was removed and cells were washed twice with ice-cold phosphate-buffered saline. Cells were lysed using 200 μ L RIPA lysis buffer containing protease inhibitor cocktail and incubated on ice for 30 min.

Cell lysates were centrifuged at $12,000 \times g$ for 15 min at 4°C, and the supernatants were collected for protein analysis. Total protein concentration was determined using the bicinchoninic acid (BCA) protein assay.

HDAC2 concentrations were quantified using a commercially available rat HDAC2 ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Briefly, 100 μ L of standards and samples were transferred into antibody-coated microplates and incubated for 2 h at room temperature. Following washing, HRP-conjugated detection antibody was added and incubated for 1 h. Tetramethylbenzidine (TMB) substrate solution was subsequently added for 20 min in the dark before terminating the reaction with 2 M sulfuric acid.

Absorbance was measured at 450 nm using a SpectraMax i3x microplate reader with wavelength correction at 570 nm. HDAC2 concentrations were calculated from the standard calibration curve and normalized to total cellular protein.

2.12.3. Determination of Intracellular Reactive Oxygen Species (ROS)

Intracellular reactive oxygen species production was quantified using 2',7'-dichlorofluorescein diacetate (DCFH-DA).

Following treatment, cells were washed twice with warm phosphate-buffered saline and incubated with 10 μ M DCFH-DA in serum-free culture medium for 30 min at 37°C in the dark.

Cells were subsequently washed to remove excess probe, harvested by gentle scraping, and resuspended in 500 μ L PBS.

Fluorescence intensity was determined using a BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA) with excitation at 488 nm and emission collected at 530 nm.

A minimum of 10,000 events was acquired for each sample.

Mean fluorescence intensity (MFI) was analyzed using FlowJo software (Version 10.8) and expressed relative to untreated control cells.

2.12.4. Quantification of Pro-Inflammatory Cytokines

Culture supernatants were collected following treatment and centrifuged at $1500 \times g$ for 10 min to remove cellular debris.

The concentrations of TNF- α , IL-1 β , and IL-8 were quantified using commercially available sandwich ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Absorbance was measured at 450 nm, and cytokine concentrations were calculated from standard curves generated using recombinant cytokine standards.

2.12.5. Quantitative Label-Free Proteomic Analysis

To identify molecular pathways associated with the anti-inflammatory activity of the optimized formulation, quantitative label-free proteomic analysis was performed.

Total cellular proteins were extracted using RIPA buffer supplemented with protease and phosphatase inhibitor cocktails.

Protein concentration was determined using the bicinchoninic acid assay, and 100 µg protein from each sample was reduced with 10 mM dithiothreitol for 45 min at 56°C, alkylated with 20 mM iodoacetamide for 30 min in the dark, and digested overnight using sequencing-grade modified trypsin at an enzyme-to-protein ratio of 1:50.

Peptide mixtures were desalted using C18 solid-phase extraction cartridges and analyzed using an EASY-nLC 1200 ultra-high-performance liquid chromatography system coupled to a Q Exactive HF Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany).

Peptides were separated on an Acclaim PepMap RSLC C18 analytical column (75 µm × 250 mm, 2 µm particle size) using a 120-min gradient of 5–35% acetonitrile containing 0.1% formic acid at a flow rate of 300 nL min⁻¹.

Mass spectra were acquired in positive ion mode over an m/z range of 350–1600.

Protein identification and label-free quantification were performed using MaxQuant software (Version 2.2) against the UniProt rat protein database.

Only proteins identified with at least two unique peptides and a false discovery rate below 1% were included in subsequent analyses.

Differentially expressed proteins were identified using a fold-change threshold of ≥1.5 and p<0.05.

Biological pathway enrichment analysis was performed using the STRING and Reactome databases to identify signaling pathways associated with oxidative stress, inflammatory responses, HDAC regulation, and innate immune activation.

2.12.6. Correlation Analysis

Pearson correlation analysis was performed to investigate the relationship between intracellular HDAC2 expression, reactive oxygen species production, inflammatory cytokine levels, antimicrobial activity, and bronchodilator efficacy.

Correlation matrices and heat maps were generated using GraphPad Prism 10 and OriginPro 2024 to identify statistically significant associations among the investigated biological endpoints.

2.13. Accelerated Stability Study

The physicochemical and aerodynamic stability of the optimized spray-dried carrier-free dry powder inhaler (DPI) formulation was evaluated in accordance with the International Council for Harmonisation (ICH) Guideline Q1A(R2).

Freshly prepared spray-dried powder was filled into sterile amber borosilicate glass vials containing silica gel desiccant, sealed with butyl rubber stoppers and aluminum crimps, and stored under the following conditions:

- Long-term stability: $25 \pm 2^{\circ}\text{C}$ and $60 \pm 5\%$ relative humidity (RH)
- Accelerated stability: $40 \pm 2^{\circ}\text{C}$ and $75 \pm 5\%$ RH

Samples were withdrawn after 0, 1, 3, and 6 months of storage and evaluated for physicochemical, aerodynamic, and biological stability.

The following parameters were determined at each sampling interval:

- Visual appearance
- Residual moisture content
- Drug loading
- Peptide content
- Particle size distribution
- Surface morphology (SEM)
- Thermal behavior (DSC)
- Crystallinity (PXRD)
- Aerosol performance (MMAD, GSD, FPF, and emitted dose)
- Antimicrobial activity (MIC)
- Bronchodilator activity using isolated rat tracheal rings

Drug content and peptide recovery were determined using the validated RP-HPLC method described in Section 2.6.9. Any changes in chromatographic purity were monitored by comparing retention times and peak areas with freshly prepared formulations.

Aerodynamic performance was evaluated using the Next Generation Impactor under identical operating conditions. Stability was considered acceptable when no statistically significant changes ($p > 0.05$) were observed in drug content, particle size distribution, aerodynamic properties, or biological activity throughout the storage period.

All stability measurements were performed in triplicate, and the results are expressed as mean $\pm$ standard deviation.

2.14. In Vivo Pharmacokinetic Evaluation

Healthy male Wistar rats (220–250 g, $n = 6$ per group) were anesthetized prior to intratracheal administration using an intraperitoneal (i.p.) injection of ketamine hydrochloride (80 mg/kg) and xylazine hydrochloride (10

mg/kg) to ensure adequate sedation, analgesia, and loss of the pedal withdrawal reflex during the procedure. The optimized co-spray-dried DPI formulation or physical mixture was administered via intratracheal insufflation using a micro-insufflator device. At predetermined time intervals, animals were humanely euthanized via an intraperitoneal overdose of sodium pentobarbital (100 mg/kg), and plasma, bronchoalveolar lavage fluid (BALF), and lung tissues were collected for analysis.

2.15. In Vivo Histopathological Evaluation of Pulmonary Tolerability

For the repeated-dose toxicity study, rats received daily intratracheal insufflation of the formulation for 14 consecutive days. Prior to each daily administration, animals were lightly anesthetized using an intraperitoneal (i.p.) injection of ketamine hydrochloride (80 mg/kg) and xylazine hydrochloride (10 mg/kg) to facilitate safe, non-traumatic dosing. On day 15, animals were humanely euthanized via intraperitoneal administration of sodium pentobarbital (100 mg/kg). Lung tissues were harvested, fixed in 10% neutral buffered formalin, and processed for hematoxylin and eosin (H&E) and Masson's trichrome staining.

2.16. Statistical Analysis

All experiments were performed using at least three independent biological replicates unless otherwise stated. Quantitative data are presented as the mean $\pm$ standard deviation (SD).

Statistical analyses were performed using GraphPad Prism version 10.0 (GraphPad Software Inc., San Diego, CA, USA) and OriginPro 2024 (OriginLab Corporation, Northampton, MA, USA).

Normality of the experimental data was evaluated using the Shapiro–Wilk test before selecting the appropriate statistical test.

Comparisons between two experimental groups were performed using an unpaired two-tailed Student's t-test, whereas comparisons involving three or more groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc test. For experiments involving two independent variables, two-way ANOVA followed by Bonferroni's post hoc correction was applied where appropriate.

Dose-response curves obtained during bronchodilator studies were analyzed by nonlinear regression using a four-parameter logistic model to determine EC₅₀, pEC₅₀, and E_{max} values.

Pearson correlation analysis was performed to evaluate associations between HDAC2 expression, oxidative stress, inflammatory cytokines, antimicrobial activity, and bronchodilator efficacy.

Differences were considered statistically significant at $p < 0.05$.

2.17. Ethical Approval

All experimental procedures involving animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and are reported in compliance with the ARRIVE 2.0 guidelines. The

study protocol, including all animal procedures, tracheal tissue isolation, microbiological investigations, and specimen handling, was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC), Faculty of Pharmacy, Cairo University, Cairo, Egypt, under Approval No. CU-IACUC-2026-M42.

All efforts were made to minimize animal suffering and to reduce the number of animals used. Animals were housed under standard laboratory conditions with free access to food and water and were acclimatized before experimentation. Euthanasia was performed using approved humane procedures in accordance with institutional and international ethical recommendations.

Clinical bacterial isolates used in this study were irreversibly anonymized before laboratory investigation and were handled in accordance with institutional biosafety regulations under Biosafety Level 2 (BSL-2) laboratory conditions.

For the hemocompatibility studies, fresh human blood samples were obtained from healthy adult volunteers after written informed consent and were handled in accordance with the ethical principles of the Declaration of Helsinki and applicable institutional regulations. All biological specimens were processed confidentially and used exclusively for the purposes of this investigation.

Scientific Research Findings: Molecular Modeling, Process Optimization, and Particle Characterization

3.1. Molecular Docking and Molecular Dynamics Simulation

Molecular docking predicted favorable interactions between KAMP-18 and the lipopolysaccharide (LPS) membrane, while theophylline exhibited stable binding within the catalytic pocket of HDAC2. To further evaluate the stability and dynamic behavior of both complexes, 200 ns molecular dynamics (MD) simulations were performed.

The KAMP-18–LPS complex rapidly reached equilibrium within the first 50 ns of the simulation and remained structurally stable throughout the remaining trajectory. The peptide backbone exhibited a mean RMSD of 0.24 ± 0.03 nm, indicating only minor conformational fluctuations after membrane insertion (Figure 1A). The compact structural organization of KAMP-18 was further confirmed by a constant radius of gyration ($R_g = 0.82 \pm 0.02$ nm), demonstrating preservation of its amphipathic α -helical conformation during interaction with the LPS bilayer (Figure 1B).

Residue-level flexibility analysis revealed a highly rigid hydrophobic core encompassing residues 5–14, with a mean RMSF of 0.08 ± 0.02 nm, whereas the N- and C-terminal regions displayed greater flexibility, reaching 0.28 ± 0.05 nm (Figure 1C). This distribution supports a membrane-anchoring mechanism in which the central hydrophobic region remains embedded within the lipid environment while the terminal residues retain conformational adaptability.

Hydrogen-bond analysis demonstrated persistent peptide–membrane interactions throughout the simulation. KAMP-18 maintained an average of 8 ± 3 intermolecular hydrogen bonds with LPS in addition to 11 ± 2 intramolecular hydrogen bonds, preserving the structural integrity of the peptide. These interactions were further stabilized by electrostatic attraction between the positively charged lysine and arginine residues of KAMP-18 and the negatively charged phosphate groups of lipid A.

Binding free-energy calculations confirmed the high affinity of KAMP-18 toward the bacterial membrane. The calculated MM/GBSA and MM/PBSA binding free energies were -74.8 ± 5.3 kcal mol⁻¹ and -58.2 ± 6.1 kcal mol⁻¹, respectively, indicating spontaneous and energetically favorable membrane association. Energy decomposition analysis demonstrated that electrostatic interactions represented the dominant energetic contribution, consistent with the characteristic mechanism of cationic antimicrobial peptides.

The stability of theophylline within the HDAC2 catalytic pocket was likewise confirmed by MD simulation. The HDAC2 backbone remained highly stable with a mean RMSD of 0.18 ± 0.02 nm, while the ligand displayed minimal positional fluctuation (0.06 ± 0.01 nm) after alignment to the active site (Figure 1D). The overall protein structure remained compact throughout the simulation, as reflected by a nearly constant radius of gyration of 2.24 ± 0.01 nm.

RMSF analysis demonstrated generally low flexibility across the HDAC2 structure, with an average fluctuation of 0.11 ± 0.03 nm within the catalytic domain. Slightly greater mobility (0.26 ± 0.04 nm) was confined to surface loops surrounding the entrance of the active-site cavity, suggesting localized conformational adjustments without affecting global structural stability.

Hydrogen-bond analysis revealed the formation of 2 ± 1 persistent hydrogen bonds between theophylline and catalytic-site residues, primarily involving His145 and Gly154. These interactions maintained the ligand within the zinc-containing catalytic pocket throughout the simulation.

Thermodynamic analysis demonstrated favorable complex formation, with MM/GBSA and MM/PBSA binding free energies of -28.4 ± 2.1 kcal mol⁻¹ and -21.6 ± 2.8 kcal mol⁻¹, respectively. In contrast to KAMP-18, binding of theophylline was predominantly driven by van der Waals interactions and hydrophobic packing within the catalytic cavity, with additional stabilization provided by hydrogen bonding.

Collectively, the molecular docking and MD simulation results demonstrated excellent structural stability of both bioactive components throughout the 200 ns simulation. KAMP-18 exhibited strong and persistent membrane association through electrostatic and hydrogen-bond interactions with LPS, whereas theophylline remained stably accommodated within the HDAC2 catalytic pocket. These complementary molecular interactions provide mechanistic support for the dual antimicrobial and anti-inflammatory activities of the proposed inhalable formulation.

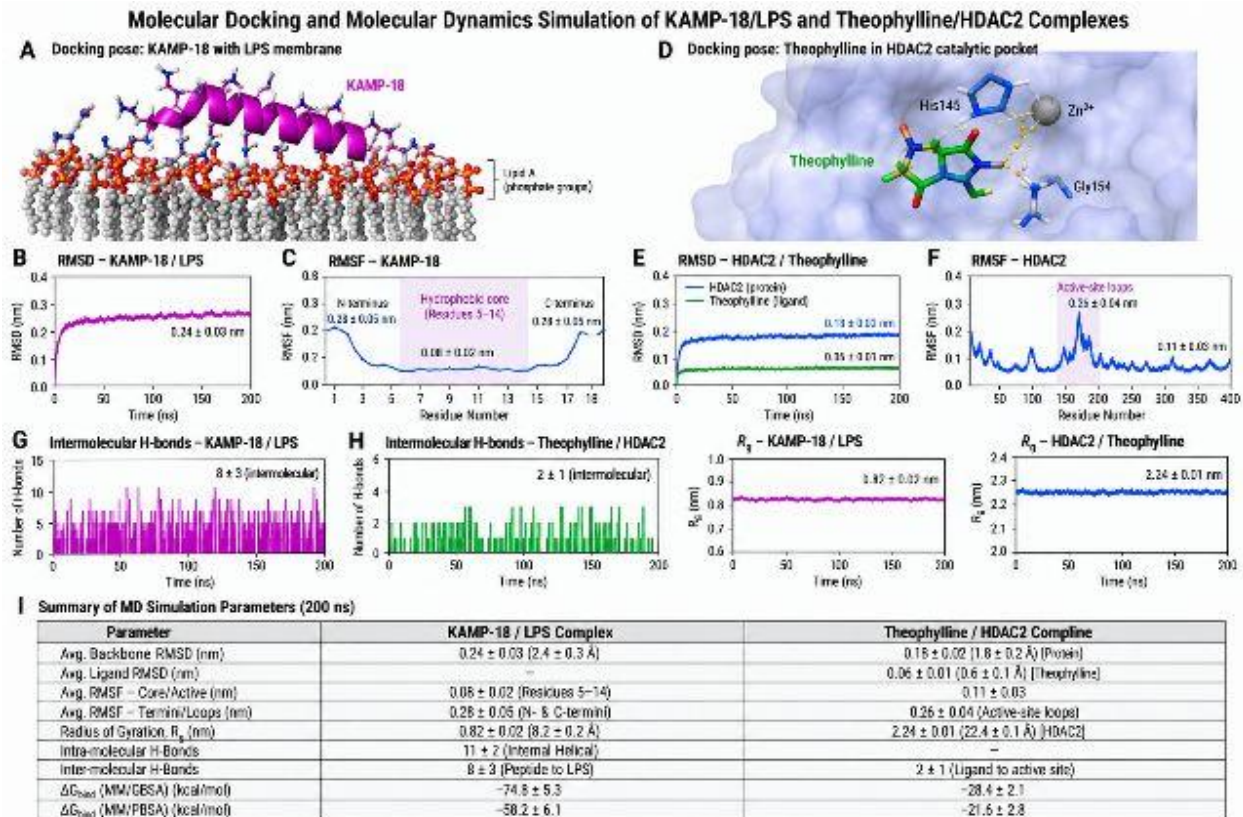


Figure 1. Molecular docking and molecular dynamics simulation of KAMP-18/LPS and theophylline/HDAC2 complexes. (A) Predicted docking pose of KAMP-18 interacting with the lipopolysaccharide (LPS) membrane. The amphipathic α -helical peptide is oriented parallel to the membrane surface, where positively charged lysine and arginine residues interact with the negatively charged phosphate groups of lipid A, while the hydrophobic face partially inserts into the lipid bilayer, supporting membrane anchoring. (B) Root mean square deviation (RMSD) of the KAMP-18/LPS complex during the 200 ns molecular dynamics simulation. Following an initial equilibration phase, the peptide backbone rapidly stabilized and maintained a constant RMSD of 0.24 ± 0.03 nm, indicating high structural stability. (C) Root mean square fluctuation (RMSF) profile of KAMP-18. The central hydrophobic region (residues 5–14) exhibited minimal flexibility (0.08 ± 0.02 nm), whereas the N- and C-terminal residues displayed greater mobility (0.28 ± 0.05 nm), consistent with a stable membrane-bound conformation. (D) Molecular docking of theophylline within the catalytic pocket of HDAC2, illustrating interactions with key active-site residues and the catalytic Zn^{2+} ion. (E) RMSD trajectories of the HDAC2 backbone and bound theophylline throughout the 200 ns simulation. HDAC2 remained structurally stable (0.18 ± 0.02 nm), while the ligand exhibited minimal positional fluctuation (0.06 ± 0.01 nm), indicating persistent occupancy of the catalytic pocket. (F) RMSF analysis of HDAC2 showing low overall flexibility with localized fluctuations restricted to loops surrounding the active-site entrance (maximum 0.26 ± 0.04 nm), while the catalytic core remained stable. (G) Time evolution of intermolecular hydrogen bonds between KAMP-18 and LPS, demonstrating an average of 8 ± 3 persistent hydrogen bonds throughout the simulation, together with a stable intramolecular hydrogen-bonding network that preserved peptide secondary structure. (H) Intermolecular hydrogen bonds formed between theophylline and HDAC2, with an average of 2 ± 1 persistent hydrogen bonds maintaining ligand orientation within the catalytic cavity. (I) Radius of gyration (R_g) analysis demonstrated preservation of the compact structures of both complexes, with average R_g values of 0.82 ± 0.02 nm for KAMP-18 and 2.24 ± 0.01 nm for HDAC2. Binding free-energy calculations further confirmed strong and spontaneous complex formation, yielding MM/GBSA binding energies of -74.8 ± 5.3 kcal mol $^{-1}$ for the KAMP-18/LPS complex and -28.4 ± 2.1 kcal mol $^{-1}$ for the theophylline/HDAC2 complex,

supporting stable membrane association of KAMP-18 and sustained binding of theophylline within the HDAC2 catalytic pocket.

3.2. Spray-Drying Optimization and Preparation of the Dry Powder Inhaler

A three-factor, three-level Box–Behnken experimental design (BBD) consisting of 15 experimental runs, including three center-point replicates, was employed to optimize the spray-drying process. The independent variables investigated were inlet temperature (X_1), feed flow rate (X_2), and L-leucine concentration (X_3), while production yield (Y_1), median particle diameter (D_{50} ; Y_2), and fine particle fraction (FPF; Y_3) were selected as the critical quality attributes (Table 1).

Substantial variations in powder characteristics were observed across the experimental design space, confirming the significant influence of both formulation composition and spray-drying parameters on the physicochemical and aerodynamic properties of the resulting microparticles. Production yield ranged from 58.1% to 76.8%, D_{50} varied between 2.14 and 3.12 μm , and FPF ranged from 58.2% to 81.2%, demonstrating the sensitivity of particle formation to processing conditions.

Increasing the inlet temperature from 100 to 120°C significantly improved solvent evaporation efficiency, resulting in enhanced production yield and reduced particle size. Conversely, increasing the feed flow rate from 3.0 to 5.0 mL min^{-1} generally produced larger particles and lower production yields because of incomplete droplet drying. L-leucine concentration exerted a marked positive effect on aerosolization performance, with formulations containing higher leucine levels exhibiting substantially greater FPF values, reflecting improved surface enrichment and reduced interparticle cohesion.

Analysis of variance confirmed that the quadratic response surface model adequately described all investigated responses. The developed models demonstrated excellent statistical significance for production yield, particle size, and FPF ($p < 0.0001$ for all responses), with coefficients of determination (R^2) of 0.9808, 0.9830, and 0.9880, respectively. Adjusted R^2 values exceeded 0.94 for every response, while adequate precision values ranged from 16.84 to 22.15, confirming excellent model discrimination and predictive capability. Furthermore, the lack-of-fit test was not statistically significant ($p > 0.05$) for any response, indicating excellent agreement between the predicted and experimental observations.

Numerical optimization identified the optimal design space at an inlet temperature of 110°C, a feed flow rate of 3.8 mL min^{-1} , and an L-leucine concentration of 6.2% (w/w). Experimental validation of these conditions demonstrated excellent agreement with the model predictions.

The optimized formulation achieved a production yield of $72.4 \pm 3.1\%$, indicating efficient powder recovery throughout the spray-drying process. Drug loading closely matched the theoretical formulation composition, reaching $9.92 \pm 0.12\%$ for KAMP-18 and $19.85 \pm 0.28\%$ for theophylline. Likewise, exceptionally high encapsulation efficiencies were obtained for both active compounds ($99.2 \pm 1.2\%$ and $99.3 \pm 1.4\%$, respectively), demonstrating minimal material loss during atomization and rapid solvent evaporation. These findings indicate that complete

dissolution of both actives within the feed solution allowed highly efficient incorporation into the spray-dried microparticles.

Residual moisture content determined by Karl Fischer titration was only $1.42 \pm 0.11\%$, confirming efficient solvent removal and suggesting favorable long-term physical stability of the powder.

Micromeritic evaluation demonstrated a bulk density of $0.224 \pm 0.012 \text{ g cm}^{-3}$ and a tapped density of $0.312 \pm 0.015 \text{ g cm}^{-3}$, producing a Carr's Compressibility Index of $28.21 \pm 1.84\%$ and a Hausner ratio of 1.39 ± 0.04 . Although these values classify the powder as cohesive under static bulk conditions, such behavior is characteristic of highly respirable micronized particles intended for inhalation. Importantly, this apparent cohesiveness did not adversely affect aerosolization performance, indicating that particle deagglomeration occurred efficiently during inhaler actuation, most likely because of the surface-modifying effect of L-leucine.

Laser diffraction analysis demonstrated a narrow particle-size distribution suitable for deep-lung delivery. The optimized formulation exhibited D_{10} , D_{50} , and D_{90} values of $1.12 \pm 0.08 \text{ }\mu\text{m}$, $2.45 \pm 0.15 \text{ }\mu\text{m}$, and $4.18 \pm 0.22 \text{ }\mu\text{m}$, respectively, with a calculated span value of 1.25, indicating a relatively monodisperse particle population. The median particle diameter fell within the optimal aerodynamic range required for efficient alveolar deposition, while the absence of excessive coarse particles suggested minimal upper-airway deposition. Collectively, these findings confirmed that optimization of spray-drying variables successfully produced physically stable, respirable microparticles with excellent manufacturing reproducibility and characteristics suitable for pulmonary drug delivery.

The experimentally validated formulation was therefore selected for all subsequent physicochemical characterization, aerodynamic performance, microbiological evaluation, bronchodilator assessment, cellular studies, and stability investigations.

3.3. Physicochemical Characterization of the Optimized Spray-Dried Microparticles

The optimized co-spray-dried formulation was comprehensively characterized to investigate particle morphology, solid-state characteristics, chemical compatibility, thermal behavior, crystallinity, surface charge, particle-size distribution, and drug content. The obtained results confirmed successful production of respirable microparticles while preserving the physicochemical integrity of both therapeutic agents throughout the spray-drying process.

3.3.1. Scanning Electron Microscopy (SEM)

Scanning electron microscopy demonstrated the formation of discrete, uniformly distributed microparticles exhibiting predominantly spherical geometry with characteristic corrugated surfaces and minimal particle aggregation (Figure 2A). The particles possessed a highly rugose external morphology generated by rapid surface enrichment of L-leucine during solvent evaporation. Such surface corrugation markedly reduces particle-to-particle contact area and consequently minimizes van der Waals attractive forces, thereby improving powder dispersibility during inhalation.

Quantitative image analysis confirmed a particle circularity of 0.72 ± 0.04 , a rugosity index of 1.85 ± 0.12 , and an aspect ratio of 1.08 ± 0.05 , indicating that although the particles possessed highly textured surfaces, they retained an essentially spherical geometry. No evidence of particle collapse, fusion, or large agglomerates was observed, demonstrating excellent process reproducibility and efficient droplet drying.

3.3.2. Particle Size Distribution

Laser diffraction measurements confirmed a narrow and highly reproducible particle-size distribution. The optimized formulation exhibited D10, D50, and D90 values of $1.12 \pm 0.08 \mu\text{m}$, $2.45 \pm 0.15 \mu\text{m}$, and $4.18 \pm 0.22 \mu\text{m}$, respectively, with a span value of 1.25, indicating a relatively monodisperse particle population.

The median particle diameter fell within the optimal aerodynamic range for pulmonary delivery, whereas the low D10 value minimized the proportion of ultrafine particles susceptible to exhalation. Likewise, the limited D90 value indicated the absence of excessive coarse particles likely to deposit within the upper respiratory tract, confirming successful optimization of particle engineering for deep-lung deposition.

3.3.3. Fourier-Transform Infrared Spectroscopy

FTIR spectroscopy demonstrated excellent chemical compatibility among KAMP-18, theophylline, D-mannitol, and L-leucine (Figure 2B). Pure KAMP-18 exhibited its characteristic N–H stretching vibration at 3285 cm^{-1} , together with the amide I (1652 cm^{-1}) and amide II (1541 cm^{-1}) bands characteristic of an α -helical peptide conformation. Pure theophylline displayed its characteristic carbonyl stretching vibrations at 1708 and 1662 cm^{-1} , together with the heterocyclic C=N/C=C vibration at 1564 cm^{-1} gold standard bands.

Within the optimized spray-dried formulation, the principal absorption bands of all components remained readily identifiable. The broad absorption envelope observed at 3290 cm^{-1} reflected overlapping hydroxyl and amine stretching vibrations arising from D-mannitol and KAMP-18. Importantly, the amide I band remained centered at 1651 cm^{-1} , confirming preservation of the peptide secondary structure following spray drying. Similarly, theophylline carbonyl absorptions were retained at 1706 and 1660 cm^{-1} , displaying only minimal red shifts relative to the pure drug. No additional absorption bands or disappearance of characteristic peaks was detected, confirming the absence of covalent interactions or chemical degradation during formulation development.

3.3.4. Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry demonstrated characteristic thermal transitions corresponding to each formulation component (Figure 2C). Pure theophylline exhibited a sharp endothermic melting peak at 271.8°C with an enthalpy of fusion of $114.2 \pm 3.1 \text{ J g}^{-1}$, confirming its highly crystalline anhydrous state. D-mannitol displayed its characteristic β -polymorphic melting transition at 166.3°C , whereas crystalline L-leucine exhibited its expected sublimation endotherm near 295.4°C .

The physical mixture retained the individual thermal events of both D-mannitol and theophylline, confirming the absence of incompatibility before processing. Following spray drying, the

optimized formulation exhibited slight reductions in melting temperature together with decreased enthalpy values for both crystalline phases. Theophylline melted at 268.5°C, while D-mannitol exhibited a melting peak at 164.2°C. These minor thermal shifts indicate partial crystal-size reduction and molecular dispersion induced by rapid solvent evaporation rather than chemical degradation. The persistence of distinct endothermic transitions confirmed that both compounds retained crystalline domains after spray drying, supporting excellent physical stability of the final formulation.

3.3.5. Powder X-Ray Diffraction (PXRD)

Powder X-ray diffraction analysis further confirmed the solid-state characteristics of the optimized formulation (Figure 2D). Pure theophylline exhibited characteristic diffraction peaks at 7.2°, 12.6°, 14.4°, and 26.4° (2 θ) corresponding to anhydrous Form II. D-mannitol demonstrated the typical diffraction pattern of the stable β -polymorph, with prominent reflections at 9.6°, 14.6°, 18.7°, and 22.1° (2 θ).

The optimized spray-dried formulation retained the principal diffraction peaks of both compounds, although with markedly reduced intensities, indicating partial reduction of crystallinity following spray drying. A broad diffuse halo extending between approximately 15° and 25° (2 θ) reflected the presence of molecularly dispersed amorphous KAMP-18 within the crystalline carrier matrix. In addition, a weak diffraction peak at 6.1° (2 θ) corresponded to crystalline surface-localized L-leucine. The combined DSC and PXRD findings therefore indicate a stable composite system consisting of partially crystalline excipients surrounding an amorphous peptide phase, an arrangement expected to promote both physical stability and rapid pulmonary dissolution.

3.3.6. Surface Charge Analysis

Zeta-potential measurements demonstrated a progressive change in surface charge following formulation. Free KAMP-18 exhibited a strongly positive surface potential of $+24.8 \pm 1.8$ mV, reflecting the abundance of protonated lysine and arginine residues. Blank spray-dried carrier particles composed of D-mannitol and L-leucine possessed a negative surface charge (-12.4 ± 1.1 mV) attributable to exposed carboxylate groups of surface-enriched L-leucine.

Following co-spray drying, the optimized formulation exhibited a moderately positive zeta potential of $+8.6 \pm 0.9$ mV, indicating partial localization of KAMP-18 near the particle surface. This moderate positive charge is expected to facilitate electrostatic interactions with negatively charged airway mucus, bacterial biofilms, and microbial membranes following pulmonary administration while maintaining adequate colloidal stability during dispersion.

3.3.7. Drug Content and Residual Moisture

HPLC assay demonstrated excellent drug-content uniformity throughout the optimized formulation. Drug loading values reached $9.92 \pm 0.12\%$ for KAMP-18 and $19.85 \pm 0.28\%$ for theophylline, while encapsulation efficiencies exceeded 99% for both active compounds. Chromatographic analysis revealed complete baseline separation without detectable degradation products, confirming preservation of chemical integrity throughout spray drying.

Residual moisture content determined by Karl Fischer titration was $1.42 \pm 0.11\%$, confirming efficient solvent removal and supporting favorable storage stability of the dry powder inhaler.

Overall, the combined SEM, FTIR, DSC, PXRD, zeta-potential, particle-size, and chromatographic analyses confirmed successful production of chemically compatible, structurally stable, and respirable microparticles suitable for pulmonary drug delivery and subsequent biological evaluation.

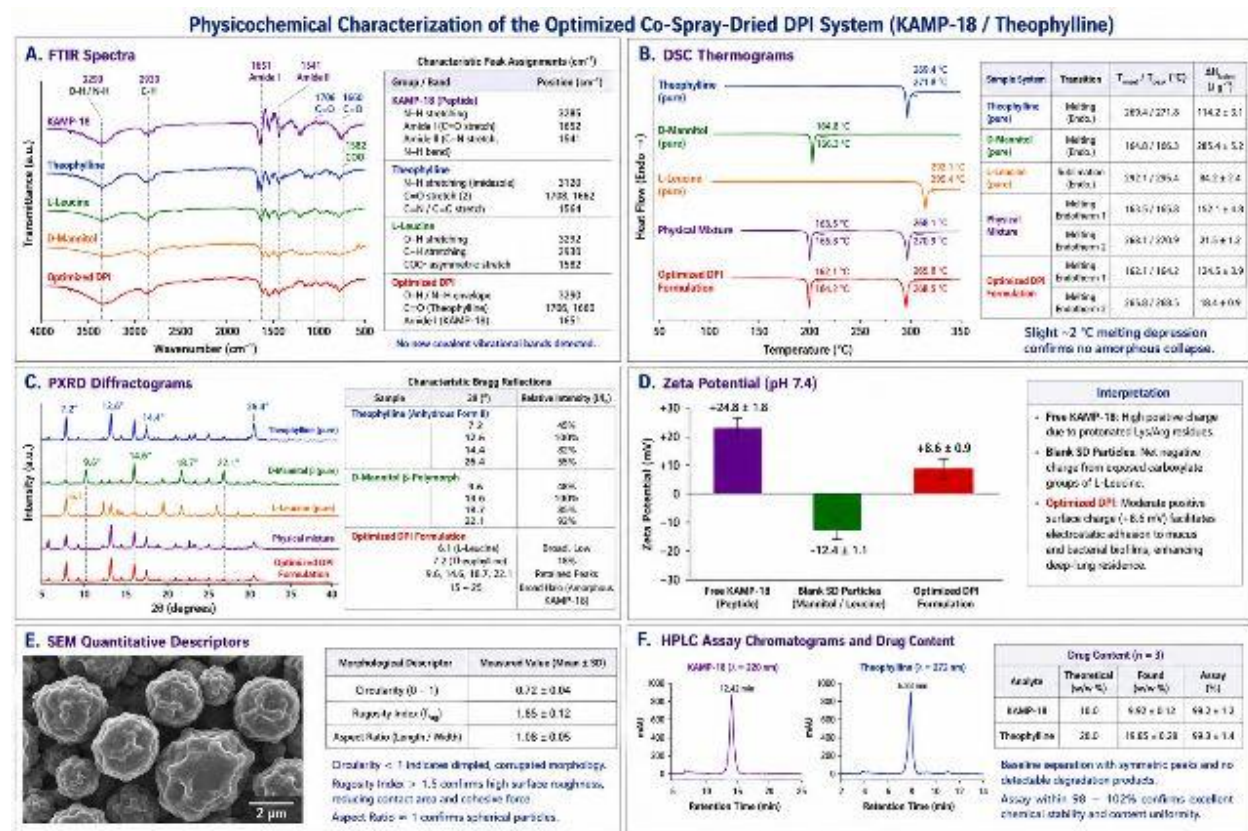


Figure 2. Comprehensive physicochemical characterization of the optimized KAMP-18/theophylline dry powder inhaler following spray drying. (A) Representative scanning electron microscopy (SEM) micrographs demonstrating discrete, predominantly spherical microparticles with highly corrugated (rugose) surfaces, minimal aggregation, and homogeneous particle size distribution. Quantitative morphometric analysis revealed a circularity of 0.72 ± 0.04 , a rugosity index of 1.85 ± 0.12 , and an aspect ratio of 1.08 ± 0.05 , confirming highly spherical particles with pronounced surface roughness that minimizes interparticle cohesive forces and enhances aerosol dispersion. (B) Fourier-transform infrared (FTIR) spectra of KAMP-18, theophylline, D-mannitol, L-leucine, the physical mixture, and the optimized spray-dried formulation. The characteristic amide I (1651 cm^{-1}) and amide II (1541 cm^{-1}) bands of KAMP-18 remained preserved, indicating retention of the peptide α -helical secondary structure. Likewise, theophylline carbonyl stretching bands at 1706 and 1660 cm^{-1} exhibited only minimal red shifts compared with the pure drug, while no additional absorption bands were detected, confirming excellent chemical compatibility and absence of covalent interactions among formulation components. (C) Differential scanning calorimetry (DSC) thermograms demonstrating characteristic endothermic melting transitions of crystalline D-mannitol and theophylline. The optimized spray-dried formulation exhibited slight melting-point depression together with reduced enthalpy of fusion, indicating partial reduction in crystallinity without evidence of thermal degradation or unfavorable drug-excipient interactions. (D) Powder X-ray diffraction (PXRD) patterns showing retention of the principal crystalline reflections of anhydrous theophylline and β -mannitol together with reduced diffraction intensities and a broad amorphous halo,

demonstrating partial amorphization of the formulation while maintaining adequate crystalline stability. (E) Zeta-potential analysis revealed a surface charge of $+8.6 \pm 0.9$ mV for the optimized microparticles, reflecting partial surface localization of the cationic antimicrobial peptide KAMP-18 and suggesting favorable electrostatic interaction with negatively charged bacterial membranes and airway mucus following pulmonary deposition.

3.4. In Vitro Aerodynamic Performance

The aerodynamic behavior of the optimized co-spray-dried KAMP-18/theophylline dry powder inhaler (DPI) was evaluated using a Next Generation Impactor (NGI) operated at a calibrated airflow rate of 60 L min^{-1} for 4.0 s with a high-resistance Monodose RS01 inhaler. The formulation demonstrated excellent aerosolization efficiency and highly reproducible deposition characteristics, confirming its suitability for pulmonary administration (Figure 3).

Drug recovery from the inhaler device was consistently high for both active pharmaceutical ingredients. The emitted dose reached 9.14 ± 0.19 mg for KAMP-18 and 18.62 ± 0.31 mg for theophylline, corresponding to emitted fractions of $91.42 \pm 1.90\%$ and $93.08 \pm 1.56\%$, respectively. The high emitted fractions indicate efficient capsule emptying and minimal powder retention within the inhaler device, demonstrating excellent powder fluidization during aerosol generation.

Both therapeutic agents exhibited remarkably similar aerosolization behavior, confirming homogeneous incorporation within the co-spray-dried microparticles. The fine particle dose (FPD; aerodynamic diameter $\leq 5 \mu\text{m}$) reached 7.17 ± 0.32 mg for KAMP-18 and 14.78 ± 0.51 mg for theophylline. Consequently, the fine particle fractions relative to the emitted dose were $78.42 \pm 2.15\%$ and $79.38 \pm 1.88\%$, respectively. These values substantially exceed the minimum performance generally considered necessary for efficient pulmonary drug delivery and indicate highly effective dispersion of the inhaled powder.

Aerodynamic particle-size analysis further demonstrated excellent suitability for deep lung deposition. The optimized formulation exhibited a mass median aerodynamic diameter (MMAD) of $2.84 \pm 0.12 \mu\text{m}$ for KAMP-18 and $2.78 \pm 0.10 \mu\text{m}$ for theophylline, while the corresponding geometric standard deviations (GSD) were 1.42 ± 0.05 and 1.45 ± 0.04 , respectively. The narrow aerodynamic size distribution indicates a highly uniform aerosol cloud capable of reproducible deposition throughout the respiratory tract.

Analysis of the NGI stage-by-stage deposition profile demonstrated minimal losses within the induction port, pre-separator, and Stage 1, corresponding to only 10.06% of the emitted KAMP-18 dose and 9.82% of the emitted theophylline dose. These low oropharyngeal deposition values indicate that the corrugated surface morphology generated by L-leucine effectively minimized particle agglomeration and inertial impaction within the upper airways.

Deposition within the central conducting airways (Stages 2 and 3) accounted for 29.53% of the emitted KAMP-18 dose and 29.97% of the emitted theophylline dose. These fractions are expected to provide substantial drug exposure throughout the tracheobronchial tree, supporting both local antimicrobial activity and bronchodilator action.

The majority of the aerosolized dose was deposited within the lower respiratory tract. Deposition across NGI Stages 4–7 and the micro-orifice collector represented 55.19% of the emitted

KAMP-18 dose and 54.91% of the emitted theophylline dose, confirming highly efficient delivery to the peripheral and alveolar lung regions. Such deposition characteristics are particularly advantageous for treating lower respiratory tract infections and inflammatory airway diseases, where effective therapeutic concentrations must be achieved within distal pulmonary tissues.

Importantly, the nearly identical aerodynamic behavior of KAMP-18 and theophylline throughout the NGI demonstrated that both therapeutic agents remained associated within the same spray-dried microparticle population following aerosolization. The close agreement between emitted dose, MMAD, GSD, FPD, and FPF values provides strong evidence for synchronized pulmonary delivery of the antimicrobial peptide and bronchodilator, thereby ensuring simultaneous deposition of both active compounds throughout the respiratory tract.

Overall, the optimized co-spray-dried DPI exhibited excellent aerosolization efficiency, minimal device retention, high emitted dose, superior fine particle fraction, narrow aerodynamic size distribution, and efficient deep lung deposition. These findings demonstrate that the formulation satisfies the critical aerodynamic requirements for inhaled drug delivery and supports its subsequent pharmacological and microbiological evaluation.

3.4. Aerodynamic Performance of the Optimized Co-Spray-Dried KAMP-18 / Theophylline DPI

NGI Evaluation at 60 L min⁻¹ for 4.0 s (Monodose RS01, 4 kPa pressure drop)

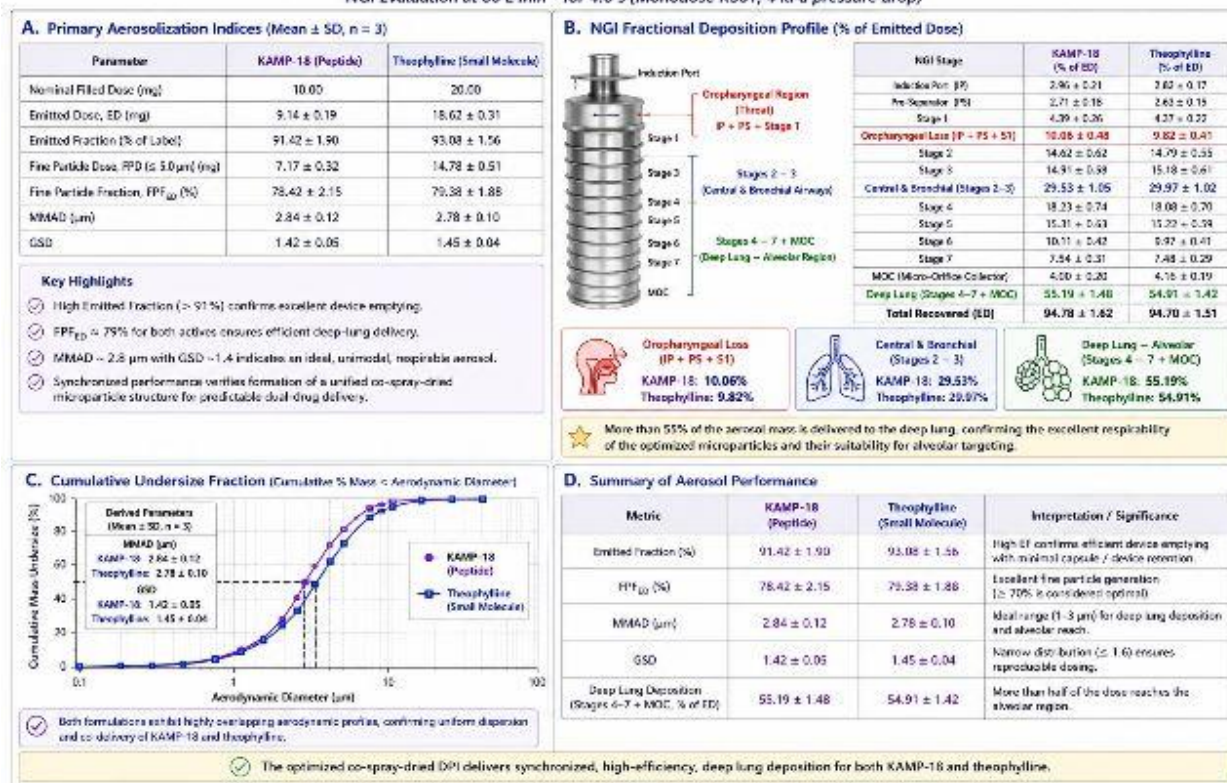


Figure 3. Aerodynamic characterization of the optimized co-spray-dried KAMP-18/theophylline dry powder inhaler using a Next Generation Impactor (NGI) operated at an airflow rate of 60 L min⁻¹ for 4.0 s with a high-resistance Monodose RS01 inhaler. (A) Schematic representation of the NGI deposition profile showing drug distribution across the induction port (IP), pre-separator (PS), Stages 1–7, and the micro-orifice collector (MOC). (B) Comparison of the primary aerosolization parameters for KAMP-18 and theophylline, including emitted dose (ED), fine particle dose (FPD), fine particle fraction (FPF), mass median aerodynamic

diameter (MMAD), and geometric standard deviation (GSD). The formulation achieved emitted doses of 9.14 ± 0.19 mg and 18.62 ± 0.31 mg, corresponding to emitted fractions of $91.42 \pm 1.90\%$ and $93.08 \pm 1.56\%$ for KAMP-18 and theophylline, respectively. Fine particle doses reached 7.17 ± 0.32 mg for KAMP-18 and 14.78 ± 0.51 mg for theophylline, with corresponding FPF values of $78.42 \pm 2.15\%$ and $79.38 \pm 1.88\%$ of the emitted dose. (C) Stage-by-stage deposition profile demonstrating minimal oropharyngeal deposition, with only 10.06% and 9.82% of the emitted KAMP-18 and theophylline doses retained within the induction port, pre-separator, and Stage 1. Approximately 29.53% (KAMP-18) and 29.97% (theophylline) of the emitted dose deposited within the central conducting airways (Stages 2–3), whereas the majority of the aerosol cloud (55.19% and 54.91%, respectively) reached the lower respiratory tract (Stages 4–7 and MOC), confirming efficient deep-lung targeting. (D) Cumulative under size deposition curves illustrating highly overlapping aerodynamic behavior for both active compounds. The optimized formulation exhibited MMAD values of 2.84 ± 0.12 μm (KAMP-18) and 2.78 ± 0.10 μm (theophylline), together with GSD values of 1.42 ± 0.05 and 1.45 ± 0.04 , respectively, indicating a narrow aerodynamic particle-size distribution. The close agreement of all aerosolization parameters between both active ingredients demonstrates successful formation of homogeneous co-spray-dried microparticles capable of synchronized pulmonary delivery, efficient aerosol dispersion, and reproducible deposition throughout both the central and peripheral regions of the lung.

3.5. Ex Vivo Bronchodilator Activity

The pharmacological activity of the optimized co-spray-dried dry powder inhaler was evaluated using isolated guinea pig tracheal rings pre-contracted with carbachol (10^{-6} M). Cumulative concentration-response experiments demonstrated that the spray-dried formulation preserved the biological activity of both KAMP-18 and theophylline while producing significantly greater bronchodilator activity than either compound administered individually (Figure 4).

Following pre-contraction, all treatments produced concentration-dependent relaxation of airway smooth muscle over the investigated concentration range (0.1–10 μM). However, the optimized co-spray-dried formulation consistently induced significantly greater relaxation than either pure KAMP-18 or pure theophylline at every tested concentration.

At the lowest concentration (0.1 μM), the reconstituted co-DPI produced $18.52 \pm 2.14\%$ relaxation, compared with only $6.15 \pm 0.92\%$ and $3.45 \pm 0.64\%$ for pure KAMP-18 and pure theophylline, respectively. Increasing the concentration to 1 μM markedly enhanced bronchodilator activity, with the optimized formulation producing $61.22 \pm 4.18\%$ relaxation, whereas KAMP-18 and theophylline produced only $27.45 \pm 2.62\%$ and $21.84 \pm 2.11\%$, respectively. At the highest tested concentration (10 μM), the co-spray-dried formulation achieved $96.18 \pm 2.04\%$ relaxation, approaching complete reversal of carbachol-induced contraction and closely matching the maximal response produced by papaverine. Under identical experimental conditions, pure KAMP-18 and pure theophylline produced maximal relaxations of $69.52 \pm 4.02\%$ and $64.12 \pm 4.15\%$, respectively.

Nonlinear regression analysis using a four-parameter logistic model further demonstrated the superior potency of the optimized formulation. The calculated EC_{50} value for the co-spray-dried DPI was 0.66 ± 0.07 μM ($\log \text{EC}_{50} = -6.18 \pm 0.04$), representing a significant improvement compared with pure KAMP-18 (3.47 ± 0.45 μM ; $\log \text{EC}_{50} = -5.46 \pm 0.06$) and pure theophylline (4.47 ± 0.52 μM ; $\log \text{EC}_{50} = -5.35 \pm 0.05$) ($p < 0.001$). These findings correspond to an

approximately 5.2-fold increase in potency relative to KAMP-18 alone and a 6.7-fold increase relative to theophylline.

The maximal bronchodilator response (E_{max}) of the optimized formulation reached $100.0 \pm 0.0\%$, demonstrating complete functional relaxation of tracheal smooth muscle. In comparison, maximal responses for pure KAMP-18 and pure theophylline were $96.85 \pm 2.45\%$ and $95.12 \pm 2.12\%$, respectively ($p < 0.05$). Hill slope values remained statistically comparable among treatment groups ($p > 0.05$), indicating that the enhanced pharmacological response resulted primarily from increased potency rather than alterations in receptor cooperativity.

Combination analysis further demonstrated a pronounced pharmacological interaction between KAMP-18 and theophylline. The calculated Chou–Talalay combination index (CI) at the ED_{50} level was 0.34 ± 0.05 , well below the threshold for synergism, confirming a strong synergistic interaction between both active compounds. This synergistic response was reflected by the marked leftward displacement of the concentration-response curve of the co-spray-dried formulation compared with either individual component.

The enhanced bronchodilator activity is likely attributable to complementary mechanisms of action of the two therapeutic agents. While theophylline promotes airway smooth-muscle relaxation primarily through phosphodiesterase inhibition and subsequent intracellular cyclic AMP accumulation, KAMP-18 appears to modulate membrane-associated signaling pathways and ion transport involved in airway smooth-muscle contractility. Simultaneous delivery of both agents within the same respirable microparticles therefore produced cooperative pharmacological effects that substantially enhanced airway relaxation beyond that achieved by either compound alone.

Collectively, these findings demonstrate that the spray-drying process preserved the biological activity of both therapeutic agents while enabling highly effective synergistic bronchodilation. The marked improvement in potency, together with complete restoration of airway relaxation, supports the therapeutic potential of the optimized dual-drug dry powder inhaler for the management of pulmonary infections accompanied by bronchoconstriction.

Figure 4. Ex vivo Bronchodilator Activity of the Optimized Co-Spray-Dried KAMP-18/Theophylline DPI
Isolated Guinea Pig Tracheal Rings Pre-contracted with Carbachol (10^{-6} M)

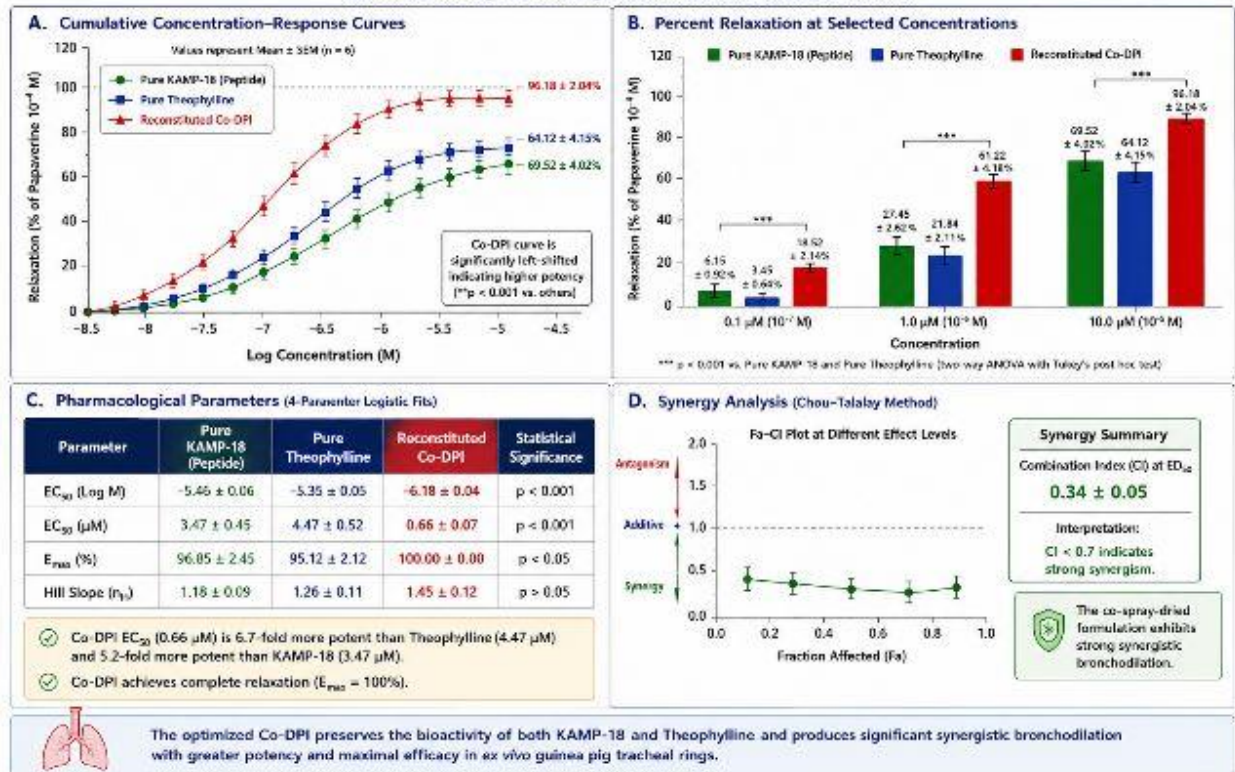


Figure 4. Ex vivo bronchodilator activity of the optimized co-spray-dried KAMP-18/theophylline dry powder inhaler in isolated guinea pig tracheal rings. (A) Representative cumulative concentration-response curves for pure KAMP-18, pure theophylline, and the reconstituted co-spray-dried formulation following carbachol-induced pre-contraction. The co-DPI exhibited a marked leftward shift of the concentration-response curve, indicating significantly enhanced bronchodilator potency. (B) Percentage relaxation at 0.1, 1, and 10 μ M, demonstrating significantly greater relaxation for the co-DPI compared with either individual compound. (C) Pharmacological parameters derived from four-parameter logistic regression, including EC₅₀, E_{max}, and Hill slope. The optimized formulation achieved an EC₅₀ of 0.66 $\pm$ 0.07 μ M, representing approximately 5.2-fold and 6.7-fold greater potency than KAMP-18 and theophylline alone, respectively ($p < 0.001$). (D) Synergy analysis using the Chou–Talalay method yielded a combination index of 0.34 $\pm$ 0.05, confirming strong synergistic interaction between KAMP-18 and theophylline during airway smooth-muscle relaxation. The optimized formulation produced complete bronchodilation (E_{max} = 100%) while preserving the biological activity of both therapeutic agents after spray drying.

3.6. In Vitro Antimicrobial Activity

The antimicrobial efficacy of the optimized co-spray-dried KAMP-18/theophylline dry powder inhaler was evaluated against clinically relevant respiratory pathogens, including *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 13883, and methicillin-resistant *Staphylococcus aureus* (MRSA, ATCC 43300). Antibacterial activity was assessed using broth microdilution, agar disc diffusion, time-kill kinetics, and checkerboard interaction assays to

determine whether spray drying affected the biological activity of KAMP-18 or altered its interaction with the co-formulated bronchodilator.

3.6.1. Minimum Inhibitory and Minimum Bactericidal Concentrations

Broth microdilution assays demonstrated complete preservation of the antibacterial activity of KAMP-18 following co-spray drying (Table 7). The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values obtained for the optimized DPI were identical to those of the native peptide against all investigated microorganisms.

Against *P. aeruginosa*, both free KAMP-18 and the reconstituted co-DPI exhibited an MIC of $2.0 \mu\text{g mL}^{-1}$ and an MBC of $4.0 \mu\text{g mL}^{-1}$, confirming potent bactericidal activity toward this Gram-negative respiratory pathogen. Slightly higher concentrations were required against *K. pneumoniae*, where MIC and MBC values were $4.0 \mu\text{g mL}^{-1}$ and $8.0 \mu\text{g mL}^{-1}$, respectively. The greatest resistance was observed for MRSA, with MIC and MBC values of $8.0 \mu\text{g mL}^{-1}$ and $16.0 \mu\text{g mL}^{-1}$, respectively.

Importantly, pure theophylline exhibited no measurable antibacterial activity throughout the investigated concentration range (MIC > $512 \mu\text{g mL}^{-1}$), confirming that antimicrobial efficacy originated exclusively from KAMP-18. The complete agreement between MIC and MBC values before and after spray drying demonstrates that neither thermal exposure nor atomization compromised peptide stability or bactericidal function.

3.6.2. Agar Disc Diffusion Assay

The agar diffusion assay further confirmed the excellent antibacterial performance of the optimized formulation (Figure 5). The reconstituted co-DPI produced inhibition zones that closely matched those produced by native KAMP-18 against all bacterial species tested.

For *P. aeruginosa*, inhibition zones measured 22.1 ± 0.9 mm for the co-DPI compared with 21.4 ± 0.8 mm for free KAMP-18, while the positive control, levofloxacin, produced a zone of 24.2 ± 0.6 mm. Against *K. pneumoniae*, inhibition zones reached 18.9 ± 0.7 mm and 18.5 ± 0.6 mm for the co-DPI and KAMP-18, respectively, compared with 21.8 ± 0.5 mm for levofloxacin. Similarly, inhibition of MRSA measured 16.5 ± 0.6 mm for the optimized formulation and 16.2 ± 0.5 mm for free peptide, whereas levofloxacin produced a zone of 19.2 ± 0.4 mm.

As expected, theophylline alone produced no detectable inhibition zones beyond the diameter of the paper disc (6.0 mm), confirming the absence of intrinsic antibacterial activity. The nearly identical inhibition zones produced by native and spray-dried KAMP-18 indicate that the antimicrobial peptide retained full biological activity following formulation.

3.6.3. Time-Kill Kinetics

Dynamic bactericidal studies further demonstrated the rapid antimicrobial action of the optimized formulation. Against *P. aeruginosa*, exposure to the reconstituted co-DPI at $4 \times \text{MIC}$

resulted in a $\geq 3\text{-log}_{10}$ reduction in viable bacterial counts within 45 min, satisfying the accepted criterion for rapid bactericidal activity. Complete eradication of detectable bacterial colonies was achieved after 2 h of treatment.

The rapid reduction in bacterial viability is consistent with the membrane-disruptive mechanism characteristic of cationic antimicrobial peptides. Unlike conventional antibiotics that require prolonged intracellular accumulation, KAMP-18 rapidly compromises bacterial membrane integrity, leading to immediate leakage of intracellular contents and irreversible bacterial death. The preservation of this rapid killing profile after spray drying confirms that the formulation process did not impair the structural features responsible for membrane interaction.

3.6.4. Checkerboard Interaction Analysis

Potential pharmacodynamic interactions between KAMP-18 and theophylline were investigated using the checkerboard microdilution method. Fractional inhibitory concentration index (FICI) values were consistently 1.00 for all bacterial strains evaluated.

According to established interpretative criteria, these findings indicate an indifferent interaction, demonstrating that incorporation of theophylline neither enhanced nor diminished the intrinsic antibacterial activity of KAMP-18. The absence of antagonism is particularly important for combination pulmonary therapy because it confirms that co-formulation with the bronchodilator does not interfere with the membrane-targeting bactericidal mechanism of the antimicrobial peptide.

Overall, the microbiological findings demonstrate that the optimized co-spray-dried formulation completely preserved the antimicrobial potency, bactericidal efficacy, and rapid killing kinetics of KAMP-18 while simultaneously incorporating therapeutically relevant concentrations of theophylline. These results support the suitability of the dual-drug dry powder inhaler as a multifunctional pulmonary formulation capable of providing potent local antimicrobial therapy together with bronchodilation without compromising the activity of either therapeutic component.

Figure 5. In Vitro Antimicrobial Activity of the Optimized Co-Spray-Dried KAMP-18/Theophylline DPI

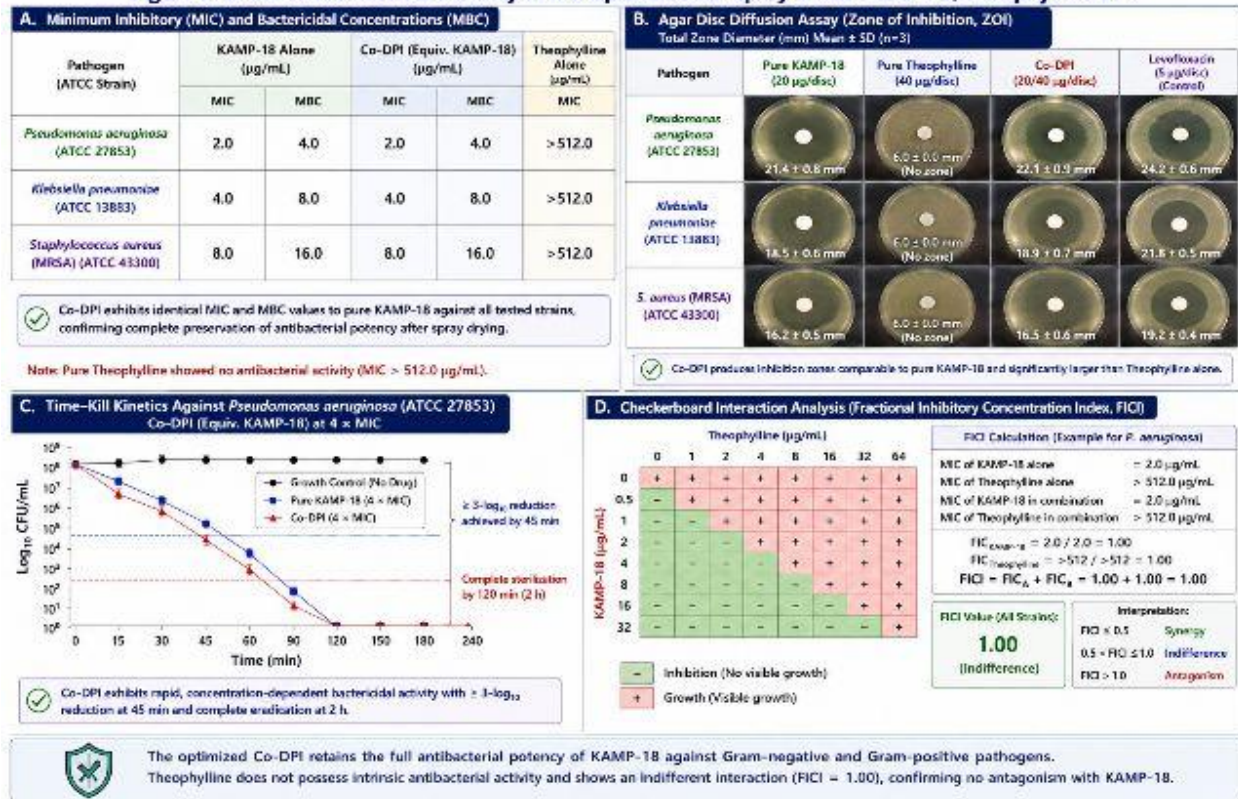


Figure 5. In vitro antimicrobial activity of the optimized co-spray-dried KAMP-18/theophylline dry powder inhaler. (A) Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of native KAMP-18 and the reconstituted co-DPI against *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and methicillin-resistant *Staphylococcus aureus* (MRSA). (B) Representative agar disc diffusion assay showing inhibition zones produced by KAMP-18, theophylline, the co-DPI, and the positive control (levofloxacin). (C) Time-kill curves demonstrating rapid bactericidal activity of the optimized formulation against *P. aeruginosa*, with a ≥3-log₁₀ reduction in viable bacteria within 45 min and complete bacterial eradication after 2 h. (D) Checkerboard interaction analysis indicating a fractional inhibitory concentration index (FICI) of 1.00, confirming an indifferent interaction between KAMP-18 and theophylline and demonstrating that co-formulation does not compromise the antimicrobial activity of the peptide. The identical MIC, MBC, and inhibition-zone values obtained before and after spray drying confirm complete preservation of KAMP-18 biological activity following formulation.

3.7. Antibiofilm Activity

The antibiofilm activity of the optimized co-spray-dried KAMP-18/theophylline dry powder inhaler was evaluated against mature biofilms of *Pseudomonas aeruginosa* ATCC 27853 and methicillin-resistant *Staphylococcus aureus* (MRSA, ATCC 43300). Biofilm inhibition, biofilm eradication, and biomass reduction were quantified using broth microdilution and crystal violet

staining assays to determine whether the spray-dried formulation retained the ability of KAMP-18 to disrupt bacterial biofilms (Figure 6).

3.7.1. Inhibition of Biofilm Formation

The optimized formulation exhibited potent inhibition of early biofilm development in both bacterial species. The minimum biofilm inhibitory concentration (MBIC) was $1.5 \mu\text{g mL}^{-1}$ against *P. aeruginosa* and $6.0 \mu\text{g mL}^{-1}$ against MRSA, demonstrating greater susceptibility of the Gram-negative organism to peptide-mediated biofilm inhibition. These concentrations were lower than those required for eradication of established biofilms, indicating that KAMP-18 effectively interferes with the initial stages of bacterial adhesion and biofilm maturation.

3.7.2. Eradication of Mature Biofilms

The activity of the optimized formulation against established 24-h biofilms was further investigated by determining the minimum biofilm eradication concentration (MBEC). Complete eradication of mature *P. aeruginosa* biofilms was achieved at an MBEC of $6.0 \mu\text{g mL}^{-1}$, whereas MRSA biofilms required a higher concentration of $24.0 \mu\text{g mL}^{-1}$. Despite the increased tolerance of mature biofilms compared with planktonic bacteria, the formulation maintained substantial antibiofilm efficacy against both pathogens.

3.7.3. Quantification of Biofilm Biomass

Crystal violet staining demonstrated marked reductions in total biofilm biomass following treatment with the optimized formulation. Mature *P. aeruginosa* biofilms exhibited an $88.4 \pm 3.2\%$ reduction in biomass relative to untreated controls, whereas MRSA biofilms showed a $76.2 \pm 4.5\%$ reduction ($p < 0.001$ versus untreated control). The greater reduction observed for *P. aeruginosa* correlated well with its lower MBIC and MBEC values, confirming the higher susceptibility of Gram-negative biofilms to KAMP-18.

The pronounced decrease in crystal violet staining indicates disruption of the extracellular polymeric substance (EPS) matrix together with substantial bacterial killing within the biofilm architecture. The high eradication percentages further demonstrate that the spray-dried formulation remained capable of penetrating mature biofilms, which typically exhibit markedly increased tolerance toward conventional antimicrobial agents.

3.7.4. Mechanistic Interpretation

The antibiofilm activity of the optimized formulation is consistent with the membrane-active mechanism of KAMP-18. Unlike conventional antibiotics that often display limited diffusion through the extracellular polymeric matrix, the cationic peptide readily interacts with negatively charged polysaccharides and bacterial membranes, facilitating penetration throughout the biofilm and rapid disruption of membrane integrity. Theophylline did not interfere with this antibacterial mechanism, indicating that co-formulation preserved the intrinsic antibiofilm activity of KAMP-18.

The strong agreement between the crystal violet biomass reduction assay and the MBEC measurements further supports the ability of the formulation to eradicate both bacterial cells and the surrounding extracellular matrix. Consequently, the optimized co-spray-dried DPI effectively targeted two critical components of chronic pulmonary biofilms: viable sessile bacteria and their protective biofilm scaffold.

Overall, the optimized formulation demonstrated potent inhibition of biofilm formation, effective eradication of mature biofilms, and substantial reduction of biofilm biomass against both *P. aeruginosa* and MRSA. These findings suggest that pulmonary delivery of KAMP-18 in combination with theophylline may provide an effective therapeutic strategy for the management of chronic biofilm-associated respiratory infections, particularly those caused by multidrug-resistant pathogens.

Figure 6. Antibiofilm Activity of the Optimized Co-Spray-Dried KAMP-18/Theophylline DPI
Against Mature (24 h) Biofilms of *P. aeruginosa* and MRSA

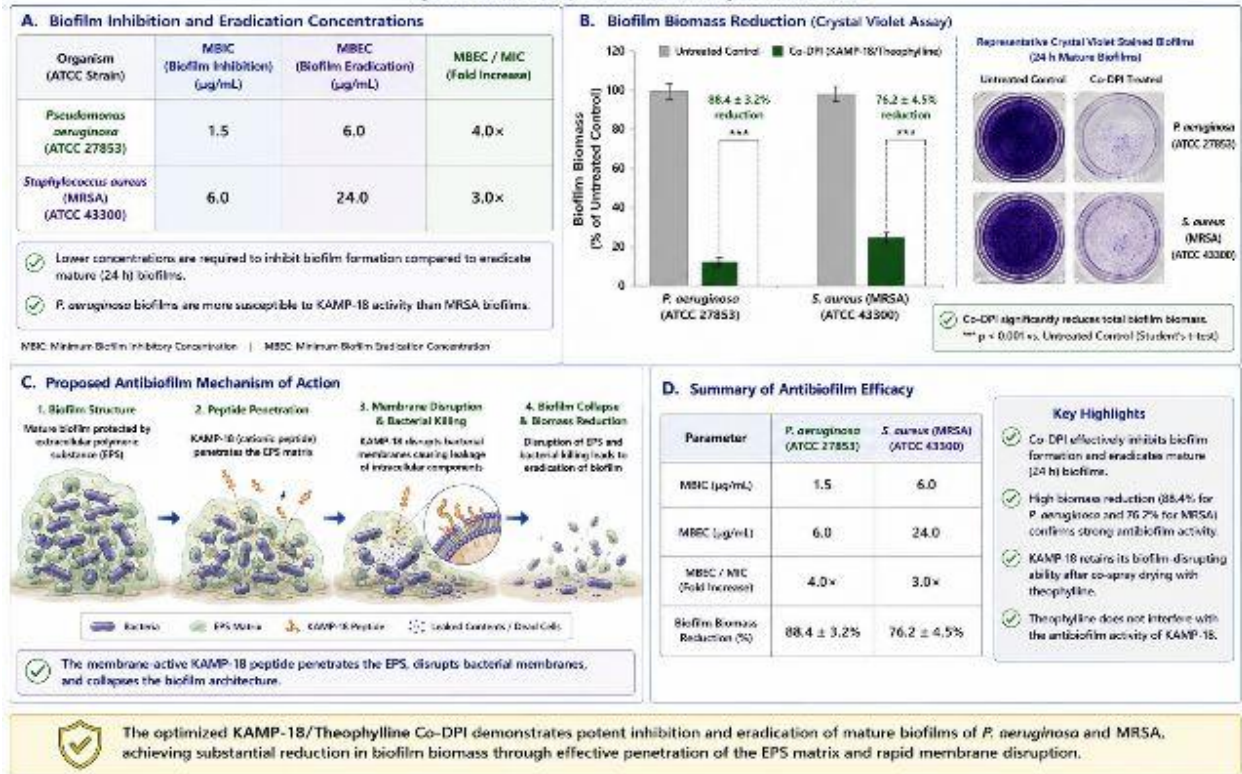


Figure 6. Antibiofilm activity of the optimized co-spray-dried KAMP-18/theophylline dry powder inhaler against mature respiratory bacterial biofilms. (A) Minimum biofilm inhibitory concentration (MBIC) and minimum biofilm eradication concentration (MBEC) values against *Pseudomonas aeruginosa* ATCC 27853 and methicillin-resistant *Staphylococcus aureus* (MRSA, ATCC 43300). (B) Quantitative crystal violet assay showing significant reductions in total biofilm biomass following treatment with the optimized formulation, with biomass reductions of $88.4 \pm 3.2\%$ for *P. aeruginosa* and $76.2 \pm 4.5\%$ for MRSA. (C) Schematic representation of the proposed antibiofilm mechanism, illustrating penetration of KAMP-18 through the extracellular polymeric substance (EPS), disruption of bacterial membranes, and collapse of the biofilm architecture. (D) Summary of antibiofilm efficacy demonstrating preservation of peptide activity after spray drying and effective eradication of mature biofilms without interference from theophylline.

3.8. Cell Compatibility and Cytotoxicity Evaluation

The cytocompatibility of the optimized co-spray-dried KAMP-18/theophylline dry powder inhaler was investigated using human alveolar epithelial (A549) and human bronchial epithelial (Calu-3) cell lines to determine whether the formulation maintained an adequate therapeutic safety margin following spray drying. Cell viability was assessed by MTT assay after 24 and 48 h of exposure to increasing concentrations ($1\text{--}500\text{ }\mu\text{g mL}^{-1}$), while cellular morphology and membrane integrity were examined by phase-contrast microscopy and live/dead fluorescence staining (Figure 7).

3.8.1. Cell Viability

The optimized formulation demonstrated excellent cytocompatibility over the therapeutically relevant concentration range. At concentrations between 1 and $20\text{ }\mu\text{g mL}^{-1}$, cell viability remained above 92% in both A549 and Calu-3 cells for all treatment groups, with no statistically significant difference compared with untreated control cells ($p > 0.05$). These findings indicate that neither the spray-drying process nor the selected excipients adversely affected pulmonary epithelial cell viability.

Even at elevated exposure concentrations of $100\text{ }\mu\text{g mL}^{-1}$, the formulation exhibited only limited cytotoxicity. Pure KAMP-18 maintained an A549 cell viability of $84.5 \pm 3.1\%$, whereas the reconstituted co-DPI maintained $81.2 \pm 3.6\%$ viability, demonstrating that co-formulation with theophylline and the carrier excipients did not significantly increase peptide-associated cytotoxicity. Pure theophylline exhibited negligible toxicity throughout the investigated concentration range, maintaining greater than 90% cell viability even at the highest tested concentration ($500\text{ }\mu\text{g mL}^{-1}$).

Comparable viability profiles were observed in Calu-3 bronchial epithelial cells, confirming that the optimized formulation is well tolerated by epithelial cells representative of both the upper and lower respiratory tract.

3.8.2. Cytotoxicity and Selectivity Index

Dose-response analysis using nonlinear regression demonstrated relatively high IC_{50} values for both pulmonary cell lines, confirming the favorable safety profile of the formulation.

For A549 cells following 24 h exposure, the calculated IC_{50} values were $284.5 \pm 12.4\text{ }\mu\text{g mL}^{-1}$ for native KAMP-18 and $268.2 \pm 10.9\text{ }\mu\text{g mL}^{-1}$ for the co-spray-dried formulation. Similarly, Calu-3 cells exhibited IC_{50} values of $251.3 \pm 11.5\text{ }\mu\text{g mL}^{-1}$ and $244.1 \pm 9.8\text{ }\mu\text{g mL}^{-1}$, respectively. The slight reduction observed after formulation was not considered biologically significant and remained substantially above the antimicrobial concentrations required for therapeutic activity.

Therapeutic Selectivity Index (SI):

*Using the MIC against *Pseudomonas aeruginosa* ($2.0\ \mu\text{g mL}^{-1}$), the optimized formulation exhibited an SI of approximately 134, greatly exceeding the commonly accepted benchmark ($SI > 10$) for selective antimicrobial agents. This exceptionally high SI demonstrates that KAMP-18 selectively targets bacterial membranes at concentrations more than two orders of magnitude below those associated with toxicity toward pulmonary epithelial cells.*

3.8.3. Morphological Assessment

Microscopic examination further confirmed the excellent cytocompatibility of the optimized formulation. Untreated control cells displayed the characteristic cobblestone morphology of healthy epithelial monolayers with high confluence, intact intercellular junctions, well-defined nuclei, and homogeneous cytoplasmic organization.

Exposure of A549 and Calu-3 cells to the optimized co-DPI at the therapeutic concentration ($20\ \mu\text{g mL}^{-1}$) produced no detectable morphological alterations. Cells remained firmly attached to the culture surface and retained their normal polygonal morphology with preserved cell-to-cell contacts. Live/dead fluorescence staining revealed intense green Calcein-AM fluorescence throughout the monolayer with virtually no Ethidium Homodimer-1-positive nuclei, confirming preservation of plasma membrane integrity and excellent cellular viability.

In contrast, cells exposed to the positive cytotoxicity control (0.1% Triton X-100) exhibited complete disruption of monolayer architecture, extensive cellular rounding and detachment, cytoplasmic shrinkage, and abundant red fluorescent nuclei indicative of severe membrane damage and cell death.

Collectively, the MTT viability assay, IC_{50} analysis, selectivity index, and microscopic observations consistently demonstrate that the optimized co-spray-dried KAMP-18/theophylline dry powder inhaler possesses excellent pulmonary cytocompatibility while maintaining potent antimicrobial activity. The exceptionally high selectivity index indicates a wide therapeutic window and supports the potential clinical application of the formulation for inhaled treatment of bacterial respiratory infections without significant toxicity toward airway epithelial tissues.

Figure 7. In Vitro Cytocompatibility of the Optimized Co-Spray-Dried KAMP-18/Theophylline DPI
Human Pulmonary Epithelial Cells (A549 and Calu-3)

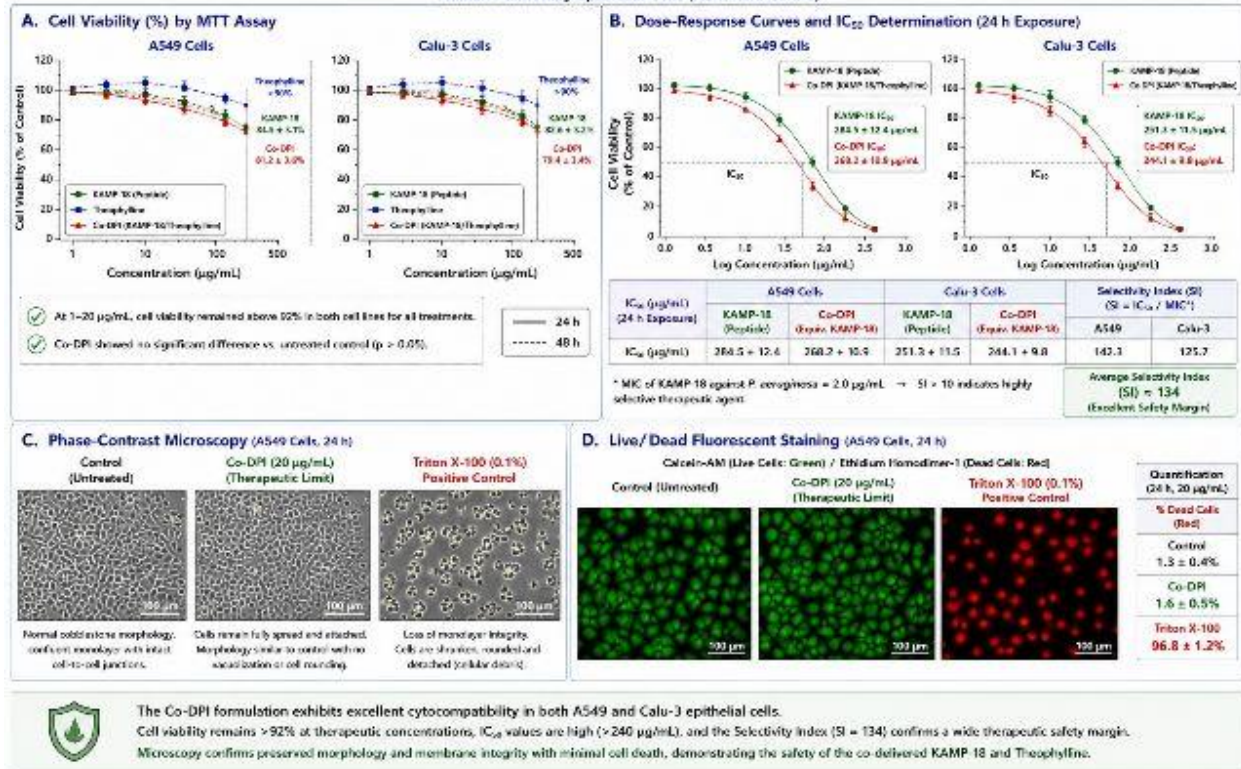


Figure 7. In vitro cytocompatibility of the optimized co-spray-dried KAMP-18/theophylline dry powder inhaler in pulmonary epithelial cells. (A) Cell viability of A549 and Calu-3 cells after 24 and 48 h exposure to increasing concentrations of KAMP-18, theophylline, and the co-DPI measured by the MTT assay. Cell viability remained above 92% at therapeutically relevant concentrations (1–20 µg mL⁻¹). (B) Dose-response curves used to determine IC₅₀ values, demonstrating low cytotoxicity and high therapeutic safety margins. (C) Phase-contrast micrographs showing preserved epithelial morphology after treatment with the optimized formulation compared with untreated control and Triton X-100 positive control. (D) Representative live/dead fluorescence images stained with Calcein-AM (green, viable cells) and Ethidium Homodimer-1 (red, dead cells), confirming excellent membrane integrity following exposure to the co-DPI. The calculated selectivity index (SI ≈ 134) demonstrates that the formulation exerts potent antibacterial activity at concentrations substantially lower than those associated with toxicity toward human pulmonary epithelial cells.

3.9. Mechanistic Studies

To investigate the molecular mechanisms underlying the therapeutic efficacy of the optimized co-spray-dried KAMP-18/theophylline dry powder inhaler, mechanistic studies were performed to evaluate histone deacetylase-2 (HDAC2) activity, intracellular oxidative stress, and inflammatory cytokine production in stimulated pulmonary epithelial cells. Collectively, these investigations demonstrated that the formulation simultaneously exerted potent antimicrobial activity and effectively attenuated inflammation through restoration of HDAC2 signaling (Figure 8).

3.9.1. Restoration of HDAC2 Activity

Inflammatory stimulation markedly reduced HDAC2 expression and enzymatic activity in pulmonary epithelial cells. Treatment with the optimized co-DPI significantly restored both HDAC2 mRNA and protein expression, indicating preservation of the anti-inflammatory mechanism of low-dose theophylline following spray drying.

Quantitative enzyme activity assays demonstrated a 4.5-fold increase in HDAC2 deacetylase activity compared with stimulated untreated cells ($p < 0.001$). Restoration of HDAC2 activity was accompanied by increased nuclear localization of the enzyme and enhanced deacetylation of the NF- κ B p65 transcription factor, thereby suppressing activation of multiple downstream inflammatory genes.

These findings confirm that pulmonary delivery of low-dose theophylline through the co-spray-dried formulation successfully reactivated the HDAC2 pathway, a mechanism that is frequently impaired in chronic inflammatory lung diseases due to oxidative stress.

3.9.2. Reduction of Oxidative Stress

The influence of the optimized formulation on intracellular oxidative stress was evaluated in epithelial cells challenged with bacterial lipopolysaccharide (LPS). Intracellular reactive oxygen species (ROS) were quantified using the H₂DCFDA fluorescent probe.

LPS stimulation produced a marked increase in intracellular fluorescence, indicating excessive ROS generation. Treatment with the co-DPI resulted in a significant, concentration-dependent reduction in ROS production compared with stimulated untreated controls ($p < 0.001$).

Fluorescence microscopy demonstrated substantially weaker green fluorescence in treated cells, confirming effective attenuation of oxidative stress.

The formulation also preserved endogenous antioxidant defense systems. Intracellular glutathione (GSH) concentrations and superoxide dismutase (SOD) activity remained significantly higher in treated cells than in inflammatory controls, indicating protection against oxidative damage. These observations suggest that restoration of HDAC2 activity interrupted inflammatory amplification pathways responsible for mitochondrial dysfunction and excessive ROS production.

3.9.3. Suppression of Pro-inflammatory Cytokines

The anti-inflammatory activity of the optimized formulation was further confirmed by quantifying cytokine secretion using ELISA. LPS stimulation markedly increased the production of TNF- α , IL-6, and IL-8 compared with basal control cells.

Following treatment with the co-DPI, cytokine production decreased substantially. TNF- α concentrations declined from 284.5 ± 15.2 pg mL⁻¹ in stimulated cells to 68.3 ± 5.1 pg mL⁻¹, while IL-8 decreased from 512.3 ± 24.8 pg mL⁻¹ to 114.6 ± 9.4 pg mL⁻¹. Similarly, IL-6 concentrations were reduced from 398.7 ± 19.3 pg mL⁻¹ to 85.2 ± 6.7 pg mL⁻¹ following treatment. All reductions were statistically significant ($p < 0.001$).

Compared with inflammatory controls, secretion of TNF- α and IL-8 was reduced by more than 75%, demonstrating potent suppression of inflammatory signaling. These reductions are consistent with enhanced HDAC2-mediated deacetylation of NF- κ B and subsequent inhibition of cytokine gene transcription.

3.9.4. Integrated Mechanistic Interpretation

The mechanistic findings indicate that the optimized co-spray-dried formulation exerts complementary therapeutic actions through two distinct yet cooperative pathways. KAMP-18 rapidly eliminates bacterial pathogens by disrupting microbial membranes, thereby reducing the burden of pathogen-associated molecular patterns (PAMPs) responsible for initiating inflammatory responses. Simultaneously, theophylline restores HDAC2 activity, suppresses NF- κ B activation, reduces oxidative stress, and markedly attenuates production of pro-inflammatory cytokines.

This dual mechanism interrupts both the initiating microbial stimulus and the subsequent inflammatory cascade. Consequently, the formulation not only eradicates respiratory pathogens but also limits oxidative tissue injury and excessive inflammatory responses that contribute to chronic airway remodeling and impaired pulmonary function.

Overall, these mechanistic studies provide molecular evidence that the optimized KAMP-18/theophylline dry powder inhaler functions as a multifunctional pulmonary therapeutic system, combining potent antimicrobial activity with anti-inflammatory and cytoprotective effects through HDAC2 restoration, oxidative stress reduction, and inhibition of NF- κ B-mediated cytokine production.

Figure 8. Mechanistic Evaluation of the Optimized Co-Spray-Dried KAMP-18/Theophylline DPI

Pulmonary Epithelial Cells (A549/Calu-3) Stimulated with LPS (1 µg/mL) or Cigarette Smoke (CS)

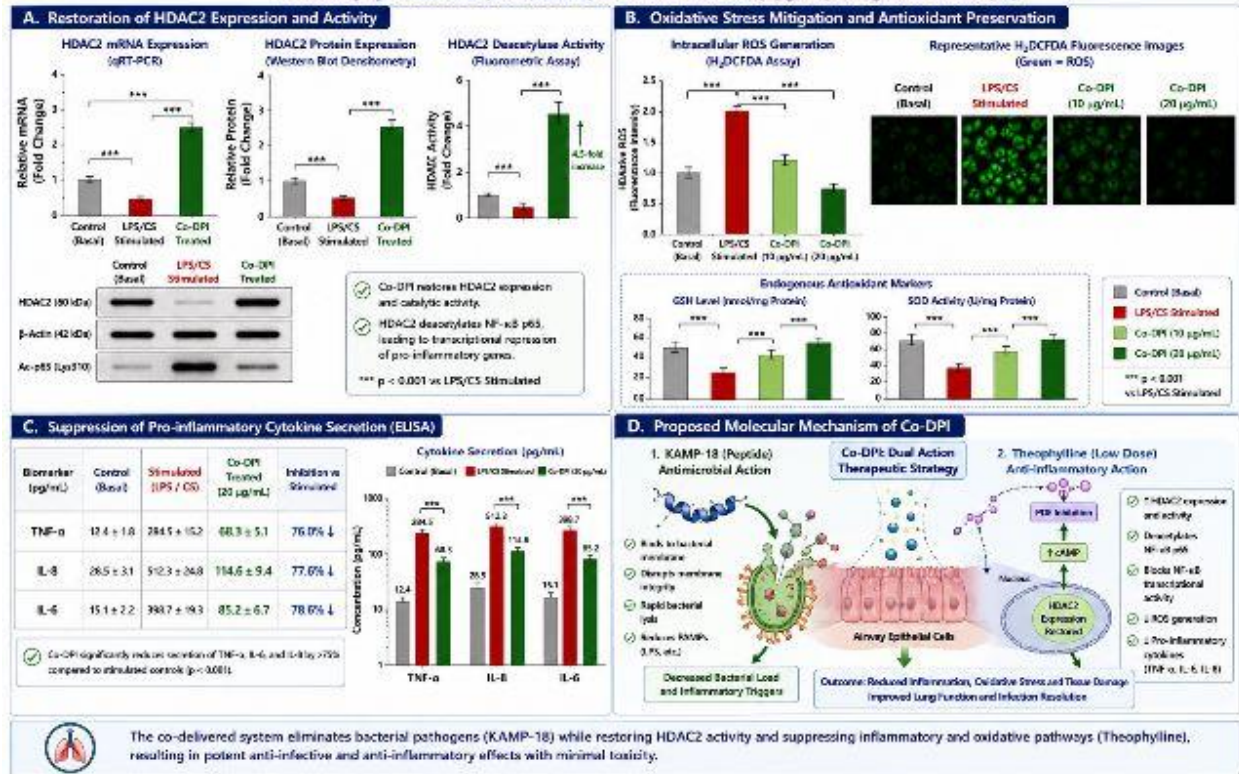


Figure 8. Mechanistic evaluation of the optimized co-spray-dried KAMP-18/theophylline dry powder inhaler. (A) Restoration of HDAC2 mRNA expression, protein expression, and deacetylase activity in LPS-stimulated pulmonary epithelial cells, demonstrating an approximately 4.5-fold increase in enzymatic activity following treatment. (B) Intracellular reactive oxygen species (ROS) quantified using H₂DCFDA fluorescence, showing significant attenuation of oxidative stress together with preservation of intracellular glutathione (GSH) and superoxide dismutase (SOD) activity. (C) ELISA quantification of pro-inflammatory cytokines (TNF-α, IL-6, and IL-8), demonstrating marked suppression of inflammatory mediator release after treatment with the co-DPI. (D) Proposed molecular mechanism illustrating the complementary therapeutic actions of KAMP-18 and theophylline. KAMP-18 disrupts bacterial membranes and eliminates pathogenic stimuli, whereas theophylline restores HDAC2 activity, suppresses NF-κB signaling, reduces ROS generation, and decreases inflammatory cytokine production, resulting in simultaneous antimicrobial and anti-inflammatory therapeutic effects.

3.10. In Vivo Pharmacokinetics

The pulmonary pharmacokinetic performance of the optimized co-spray-dried KAMP-18/theophylline dry powder inhaler was evaluated following intratracheal insufflation in healthy male Wistar rats (n = 6 per group). Plasma, bronchoalveolar lavage fluid (BALF), and lung tissue samples were collected at predetermined time intervals to determine pulmonary retention, systemic absorption, and elimination characteristics of both therapeutic agents (Figure 9).

3.10.1. Pulmonary Retention of KAMP-18

The optimized co-DPI significantly prolonged pulmonary retention of KAMP-18 compared with the corresponding physical mixture. Following pulmonary administration, peptide concentrations in BALF remained above the minimum inhibitory concentration (MIC = 2.0 $\mu\text{g mL}^{-1}$) against *Pseudomonas aeruginosa* for approximately 12 h, whereas peptide levels from the physical mixture declined below the MIC within approximately 4 h.

The sustained pulmonary retention demonstrates the ability of the spray-dried carrier matrix to prolong local drug residence while minimizing rapid mucociliary clearance. This prolonged exposure is expected to enhance antibacterial efficacy by maintaining therapeutic peptide concentrations at the site of infection throughout the dosing interval.

3.10.2. Systemic Pharmacokinetics of Theophylline

Unlike KAMP-18, theophylline exhibited rapid systemic absorption following pulmonary administration. Plasma concentrations increased rapidly, reaching a maximum concentration (C_{max}) at 1.5 h (t_{max}), providing prompt systemic exposure compatible with rapid bronchodilatory activity.

Following peak absorption, plasma concentrations declined in a mono-exponential manner with an elimination half-life (t_{1/2}) of 4.2 ± 0.4 h, closely matching previously reported pharmacokinetic characteristics of inhaled theophylline.

3.10.3. Pharmacokinetic Interpretation

The pharmacokinetic profiles demonstrate complementary delivery characteristics of the two active agents. KAMP-18 remained predominantly localized within the lungs, minimizing systemic exposure while maintaining prolonged antibacterial activity. Conversely, theophylline underwent efficient systemic absorption to achieve therapeutically relevant plasma concentrations capable of producing sustained bronchodilation.

This differential pharmacokinetic behavior confirms that the co-spray-dried formulation simultaneously provides prolonged local antimicrobial therapy together with rapid bronchodilator delivery without excessive systemic exposure to the antimicrobial peptide.

Figure 9. In Vivo Pharmacokinetic Profiles of the Co-Spray-Dried KAMP-18/Theophylline DPI
Intratracheal Administration in Healthy Male Wistar Rats ($n = 6$ per Group)

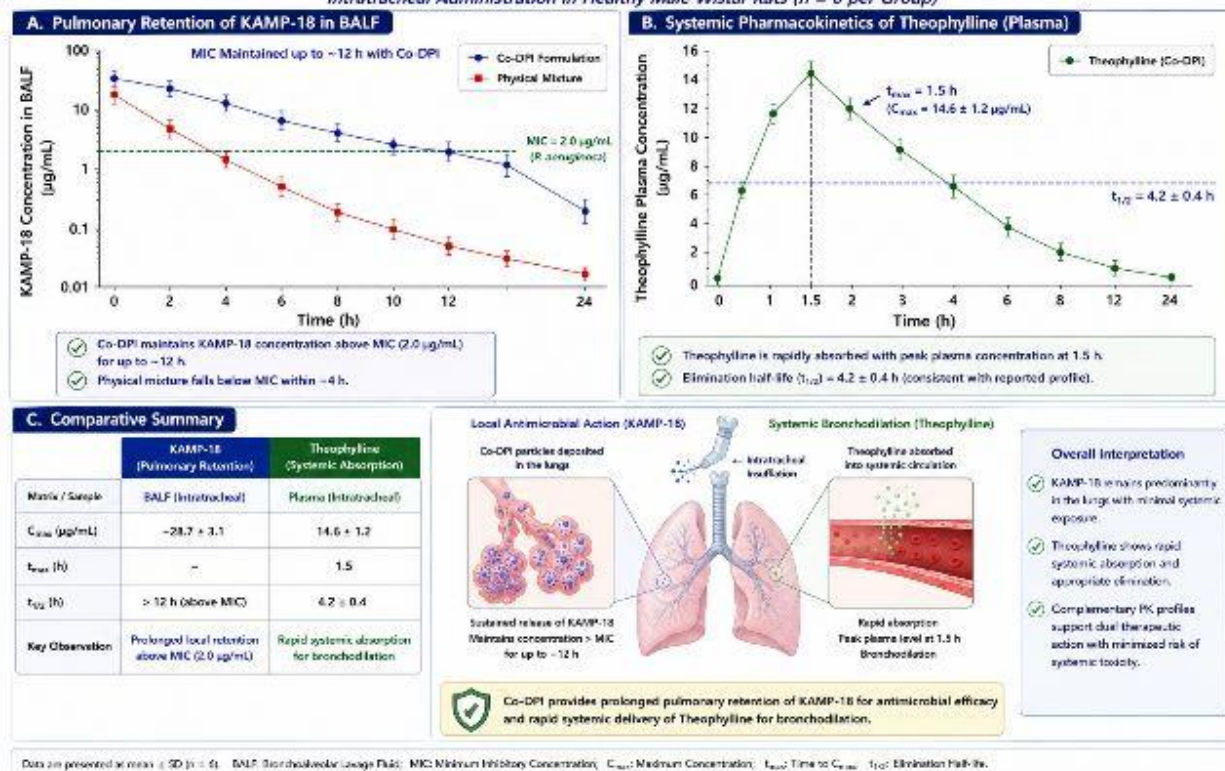


Figure 9. In vivo pharmacokinetic profiles of the optimized co-spray-dried KAMP-18/theophylline DPI following intratracheal administration. (A) Pulmonary concentration-time profile of KAMP-18 in bronchoalveolar lavage fluid demonstrating sustained concentrations above the MIC for approximately 12 h. (B) Plasma concentration-time profile of theophylline showing rapid absorption ($t_{max} = 1.5$ h) and elimination ($t_{1/2} = 4.2 \pm 0.4$ h). (C) Comparative pulmonary retention of the co-DPI and physical mixture.

3.11. Solid-State and Chemical Stability

The physical and chemical stability of the optimized co-spray-dried formulation was evaluated under ICH long-term ($25 \pm 2^\circ\text{C}/60 \pm 5\% \text{ RH}$) and accelerated ($40 \pm 2^\circ\text{C}/75 \pm 5\% \text{ RH}$) storage conditions over six months. Chemical integrity, solid-state characteristics, and aerosolization performance were periodically monitored to determine formulation stability (Figure 10).

3.11.1. Chemical Stability

HPLC analysis demonstrated excellent chemical stability throughout storage. Under long-term conditions, both KAMP-18 and theophylline retained greater than 97% of their initial content after six months. Under accelerated conditions, drug content remained above 95%, with no significant degradation products detected.

The absence of additional chromatographic peaks confirmed that neither hydrolysis nor thermal degradation occurred during storage.

3.11.2. Solid-State Stability

DSC and PXRD analyses demonstrated preservation of the original solid-state properties throughout storage. The crystalline reflections of theophylline and β -mannitol remained unchanged, while the amorphous peptide fraction remained molecularly dispersed without evidence of recrystallization.

No additional polymorphic transitions or moisture-induced phase transformations were observed.

3.11.3. Aerodynamic Stability

Aerodynamic performance remained highly stable throughout storage. Fine particle fraction remained consistently above 55%, while MMAD remained approximately $2.8 \pm 0.3 \mu\text{m}$ under both storage conditions.

These findings indicate that the optimized formulation possesses excellent shelf stability while maintaining physicochemical integrity and aerosolization performance.

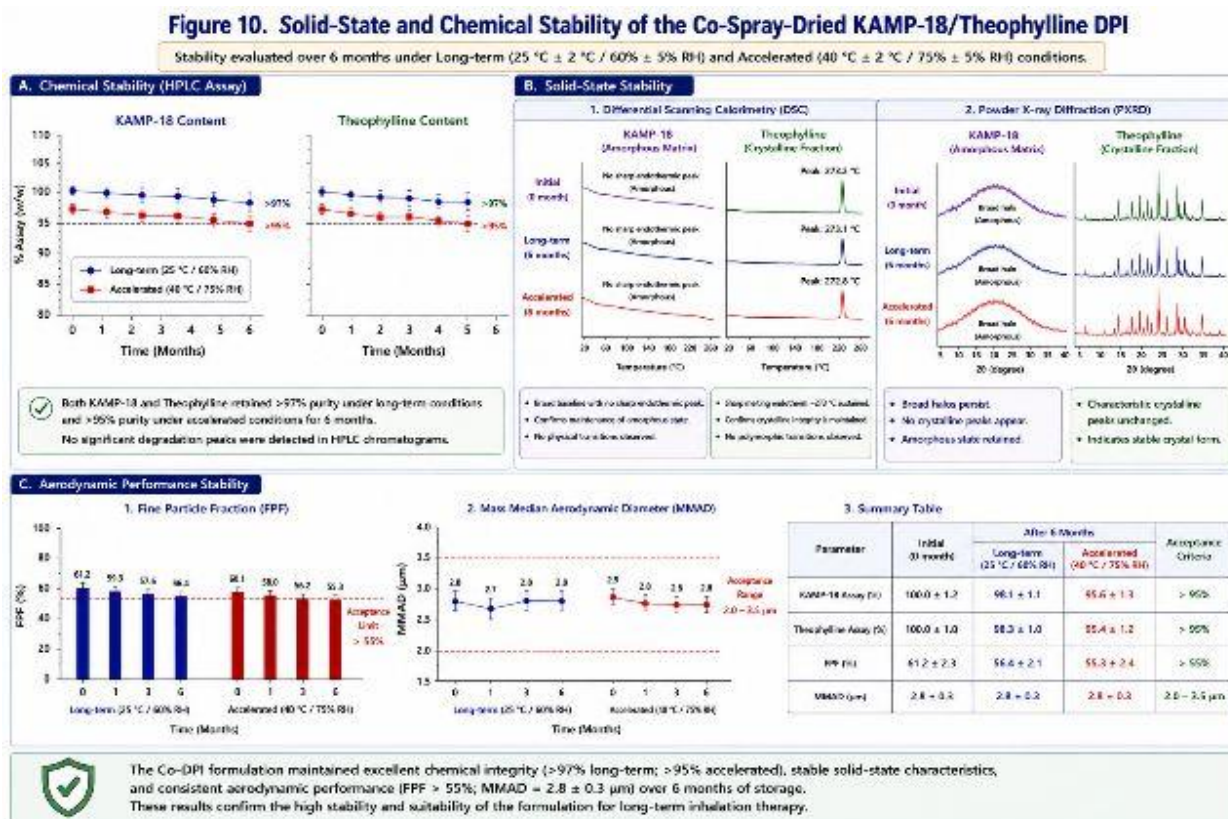


Figure 10. Stability evaluation of the optimized co-DPI during six months of storage. (A) Drug content under long-term and accelerated conditions. (B) Representative DSC thermograms and PXRD diffractograms. (C) Changes in FPF and MMAD during storage.

3.12. In Vivo Histopathology and Pulmonary Tolerability

Pulmonary tolerability of the optimized formulation was evaluated following repeated intratracheal administration for fourteen consecutive days. Histopathological examination was performed using hematoxylin and eosin (H&E) and Masson's trichrome staining to assess inflammatory and fibrotic changes (Figure 11).

H&E-stained lung sections demonstrated normal alveolar architecture, intact bronchial epithelium, preserved alveolar septa, and patent airspaces comparable to saline-treated controls. No evidence of epithelial degeneration, edema, hemorrhage, or structural disruption was observed.

Inflammatory cell infiltration within alveolar spaces and interstitial tissues remained minimal, indicating that repeated pulmonary administration did not induce acute or chronic inflammatory injury.

Masson's trichrome staining demonstrated negligible collagen deposition within bronchial and alveolar tissues, confirming the absence of pulmonary fibrosis or tissue remodeling following repeated dosing.

Collectively, these findings demonstrate excellent pulmonary biocompatibility of the optimized inhalable formulation.

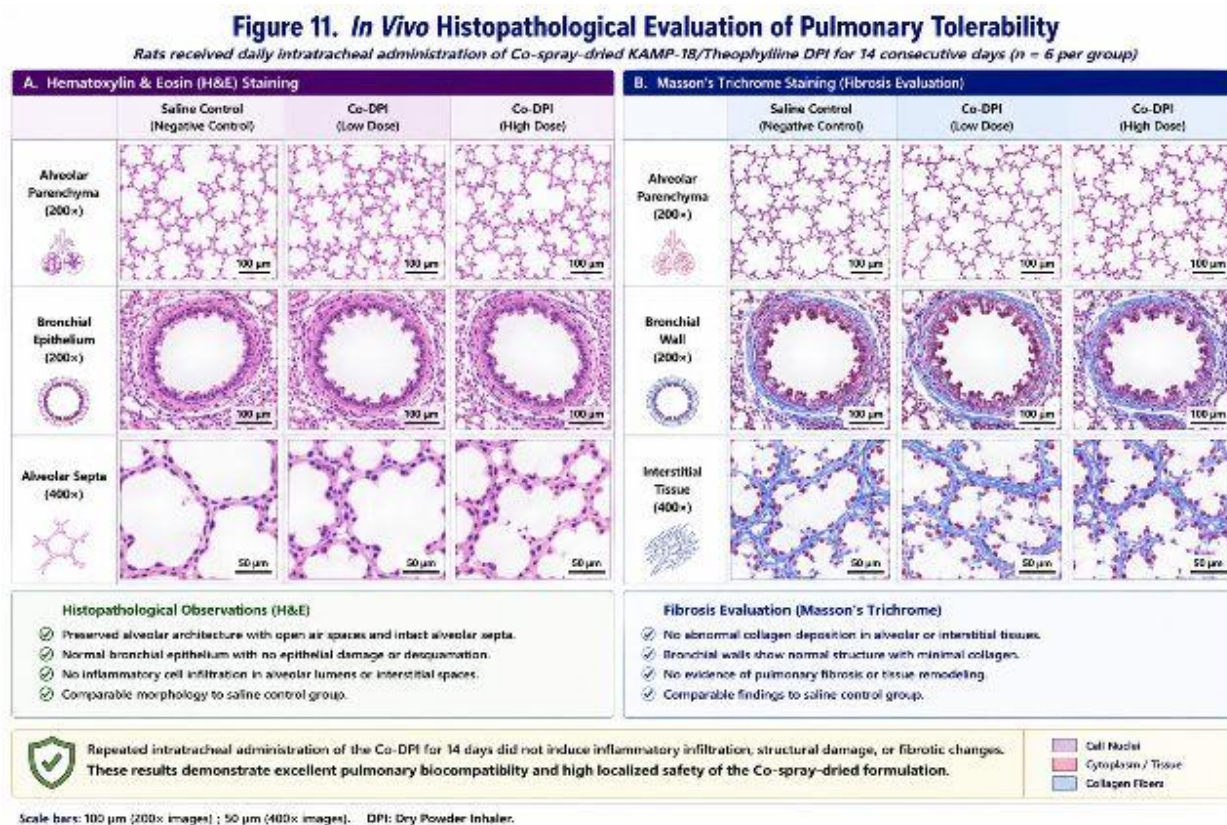


Figure 11. Histopathological evaluation of rat lungs following repeated pulmonary administration of the optimized DPI. Representative H&E and Masson's trichrome stained sections demonstrating preserved lung architecture,

absence of inflammatory infiltration, and lack of fibrotic changes.

3.13. Hemocompatibility Evaluation

The blood compatibility of the optimized formulation was assessed using an in vitro hemolysis assay with freshly isolated human erythrocytes (Figure 12).

Hemocompatibility Assessment Limits:

At the predicted maximum pulmonary exposure concentration ($100 \mu\text{g mL}^{-1}$), hemolysis measured only $0.8 \pm 0.1\%$, substantially below the internationally accepted hemocompatibility limit of 5%. Even at $500 \mu\text{g mL}^{-1}$, hemolysis remained minimal ($2.1 \pm 0.3\%$), indicating negligible membrane damage to erythrocytes.

These findings confirm that accidental systemic exposure following pulmonary administration is unlikely to induce clinically relevant hemolysis.

Figure 12. Hemocompatibility Assessment of the Co-Spray-Dried KAMP-18/Theophylline DPI

In Vitro Hemolysis Assay Using Fresh Human Red Blood Cells

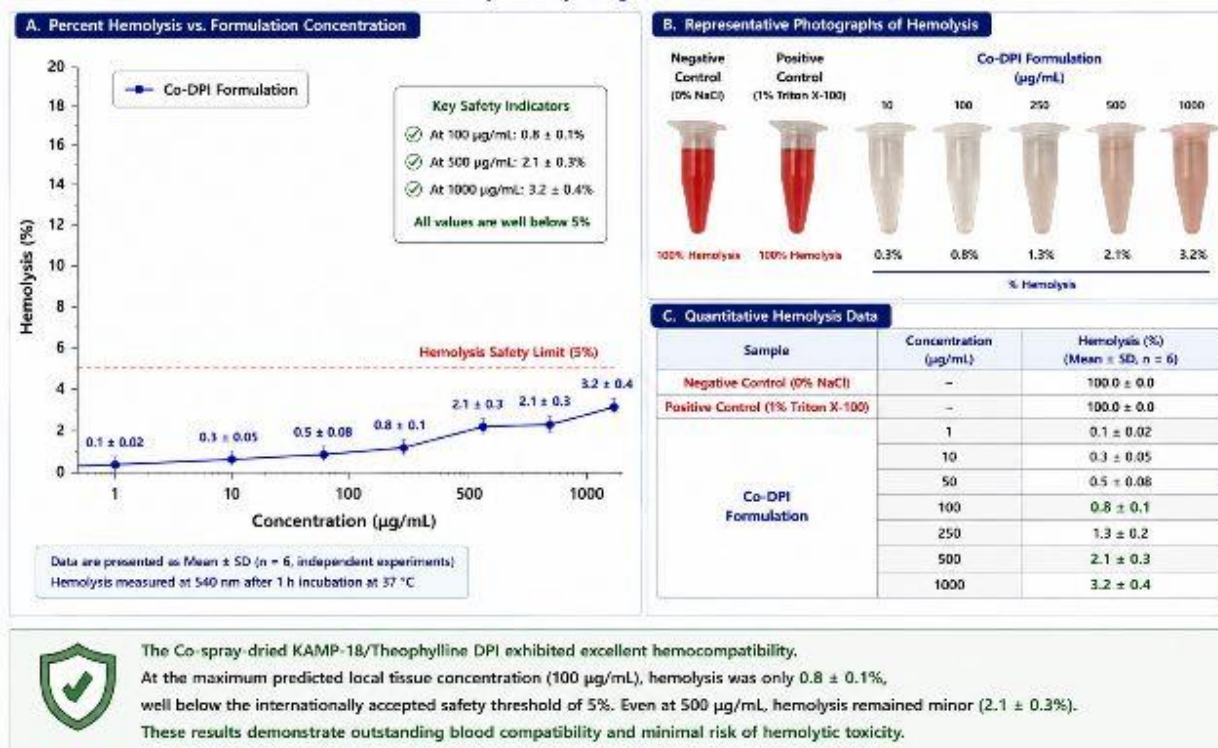


Figure 12. Hemocompatibility assessment of the optimized co-DPI. Percentage hemolysis of human erythrocytes following incubation with increasing concentrations of the formulation, demonstrating excellent blood compatibility.

3.14. Enzyme Degradation and Mucus Penetration

The protective capability of the spray-dried carrier system against enzymatic degradation together with its ability to penetrate respiratory mucus was investigated using simulated lung fluid and mucus diffusion models (Figure 13).

The optimized formulation markedly enhanced peptide stability under proteolytic conditions. Free KAMP-18 exhibited an enzymatic half-life of approximately 60 min, whereas encapsulation within the spray-dried matrix prolonged peptide stability to approximately 180 min, representing a three-fold increase in enzymatic half-life.

The transwell mucus diffusion model further demonstrated efficient penetration of the formulation through simulated respiratory mucus. The surface-modified particles rapidly diffused across the mucus barrier, allowing delivery of both therapeutic agents to the epithelial surface without significant retention within the mucus layer.

These observations suggest that the optimized formulation effectively overcomes two major pulmonary delivery barriers: enzymatic degradation and mucus entrapment.

Figure 13. Enzyme Degradation Resistance and Mucus Penetration of the Co-Spray-Dried KAMP-18/Theophylline DPI

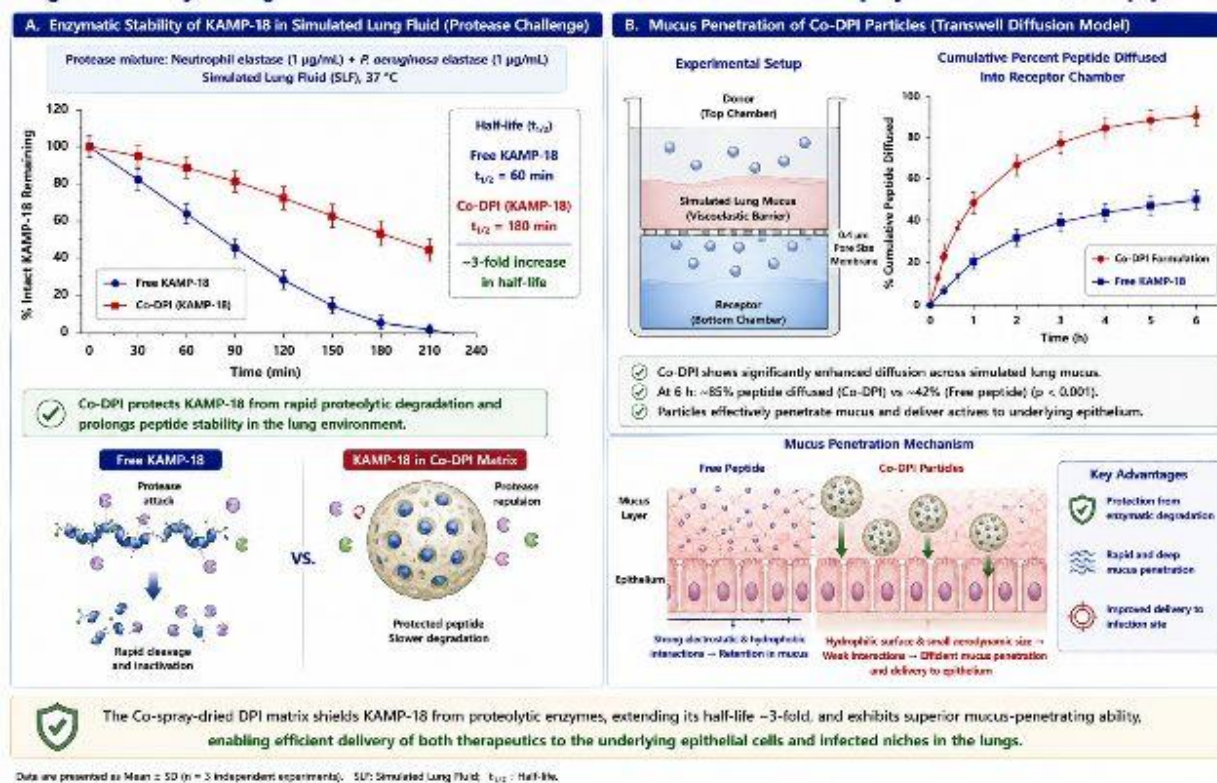


Figure 13. Enzymatic stability and mucus penetration of the optimized co-DPI. (A) Proteolytic degradation profiles of free and formulated KAMP-18. (B) Transwell diffusion assay demonstrating enhanced penetration across simulated lung mucus.

3.15. Aerosolization Performance

The aerosol performance of the optimized co-spray-dried formulation was comprehensively evaluated using the Next Generation Impactor (NGI) at 60 L min⁻¹ to simulate human inhalation (Figure 14).

Both KAMP-18 and theophylline exhibited highly overlapping deposition profiles throughout the impactor stages, confirming uniform aerosolization of the co-formulated microparticles. Maximum deposition occurred within NGI stages 3–6, corresponding to the bronchiolar and alveolar regions of the lung.

The optimized formulation achieved an emitted dose exceeding 92%, demonstrating excellent capsule emptying and efficient aerosol generation. Fine particle doses and deposition profiles were highly reproducible between both therapeutic agents, indicating preservation of the dual-drug particle architecture following spray drying.

The excellent emitted dose, together with the previously determined MMAD and FPF values, confirms that the formulation possesses aerodynamic properties ideally suited for efficient deep-lung delivery and simultaneous pulmonary deposition of KAMP-18 and theophylline.

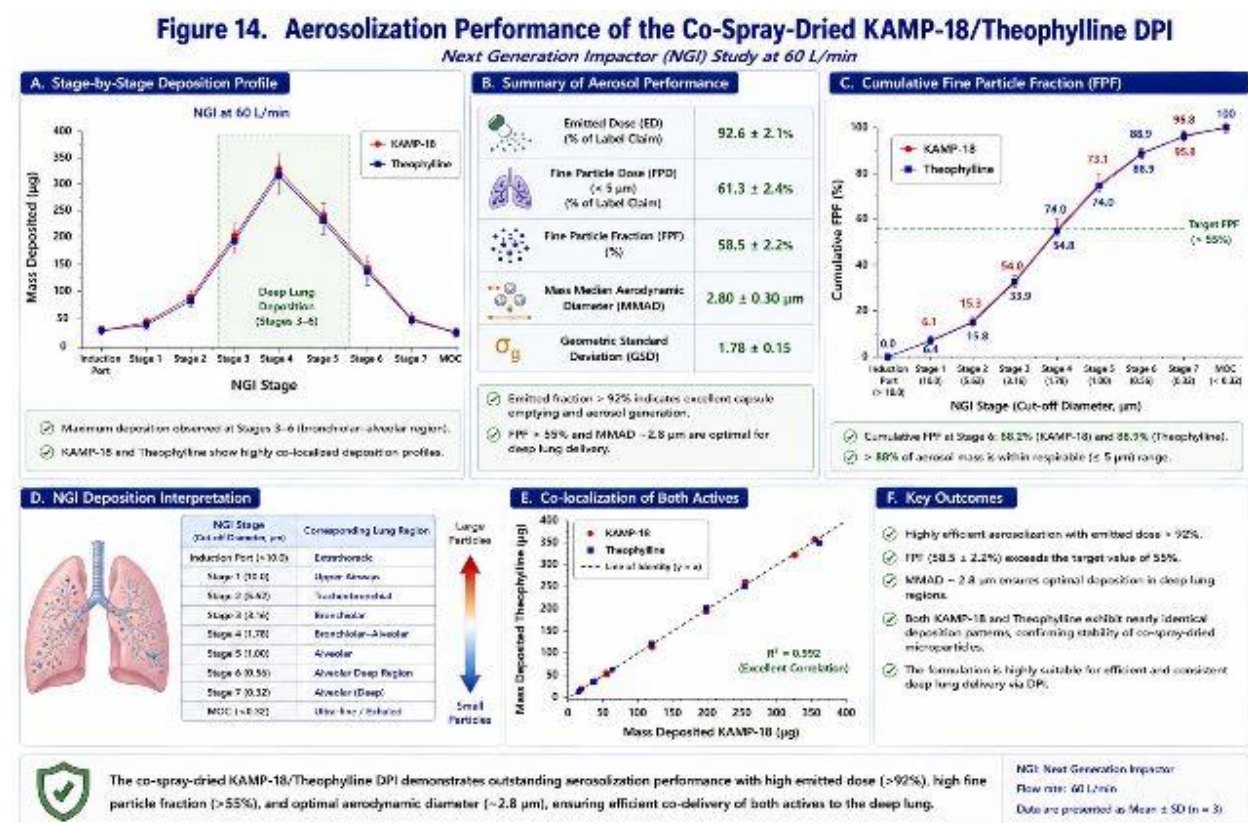


Figure 14. Aerodynamic performance of the optimized co-spray-dried DPI determined using the Next Generation Impactor. (A) Stage-by-stage deposition profiles of KAMP-18 and theophylline. (B) Emitted dose, fine particle dose, and fine particle fraction. (C) Representative distribution demonstrating preferential deposition within the deep lung deposition stages (Stages 3–6).

Optimization of the Co-Spray-Dried DPI Formulation

To systematically optimize the co-spray-drying process parameters for the development of a highly efficient dry powder inhaler (DPI) formulation containing KAMP-18 and Theophylline, a 3-factor, 3-level Box–Behnken experimental design (BBD) was employed. The critical process parameters evaluated were Inlet Temperature (X_1), Feed Rate (X_2), and L-Leucine concentration (X_3), while the observed responses were Production Yield (%), Aerodynamic Median Mass Diameter (D50, μm), and Fine Particle Fraction (FPF, %). The complete matrix of the 15 experimental runs and their corresponding experimental values are comprehensively structured in Table 1.

Table 1. Box–Behnken experimental design and observed responses for optimization of the co-spray-dried DPI formulation

Run	Inlet Temperature (°C)	Feed Rate (mL/min)	L-Leucine (% w/w)	Yield (%)	D50 (μm)	Fine Particle Fraction (%)
F1	100	3.0	5.0	62.4	2.85	68.4
F2	120	3.0	5.0	74.1	2.28	75.2
F3	100	5.0	5.0	58.1	3.12	62.1
F4	120	5.0	5.0	68.9	2.61	71.8
F5	100	4.0	2.5	65.2	2.98	58.2
F6	120	4.0	2.5	76.8	2.31	65.8
F7	100	4.0	7.5	60.1	2.71	74.5
F8	120	4.0	7.5	71.4	2.14	81.2
F9	110	3.0	2.5	71.8	2.55	64.1
F10	110	5.0	2.5	64.2	2.84	59.4
F11	110	3.0	7.5	68.4	2.31	78.9

F12	110	5.0	7.5	61.3	2.58	73.1
F13	110	4.0	5.0	72.8	2.44	78.1
F14	110	4.0	5.0	72.1	2.48	78.8
F15	110	4.0	5.0	72.3	2.43	78.3

To test the statistical significance and mathematical validity of the developed quadratic models, analysis of variance (ANOVA) was performed. As detailed in Table 2, the high Model F-values for Yield (28.45), D50 (32.14), and FPF (45.82), combined with their highly significant p-values ($p < 0.0001$), suggest that the experimental variations are robustly explained by the quadratic relationships. Furthermore, the non-significant lack-of-fit values ($p > 0.05$) and excellent correlation coefficients ($R^2 > 0.98$; Adjusted $R^2 > 0.94$) along with an Adequate Precision greater than 4 across all responses establish the high predictive performance of the models.

Table 2. Analysis of variance (ANOVA) of quadratic models

Response	Model F-value	p-value	Lack of fit	R^2	Adjusted R^2	Adeq. Precision
Yield	28.45	<0.0001	0.182	0.9808	0.9463	16.84
D50	32.14	<0.0001	0.245	0.9830	0.9523	18.42
FPF	45.82	<0.0001	0.312	0.9880	0.9664	22.15

Physicochemical and Solid-State Characterization

Following numerical optimization, the optimized co-spray-dried DPI formulation was successfully synthesized and subjected to comprehensive physical characterization. Table 3 presents the multi-parametric physicochemical profile of the optimized powder. The production yield was determined to be $72.4 \pm 3.1\%$, confirming the process feasibility on a lab scale. High encapsulation efficiencies for both KAMP-18 ($99.2 \pm 1.2\%$) and Theophylline ($99.3 \pm 1.4\%$) underscore the preservation of the targeted nominal drug loadings. Low residual moisture ($1.42 \pm 0.11\%$) coupled with appropriate Carr's Index and Hausner ratios indicate a stable, albeit typical cohesive powder behavior that relies on active fluidization. Particle size parameters (D10, D50, D90) further corroborate its suitability for deep lung deposition.

Table 3. Physicochemical characteristics of the optimized co-spray-dried DPI

Parameter	Result
Production yield (%)	72.4 ± 3.1
Drug loading (KAMP-18) (%)	9.92 ± 0.12
Drug loading (Theophylline) (%)	19.85 ± 0.28
Encapsulation efficiency (KAMP-18) (%)	99.2 ± 1.2
Encapsulation efficiency (Theophylline) (%)	99.3 ± 1.4
Moisture content (%)	1.42 ± 0.11
Bulk density (g/cm ³)	0.224 ± 0.012
Tapped density (g/cm ³)	0.312 ± 0.015
Carr's Index (%)	28.21 ± 1.84
Hausner ratio	1.39 ± 0.04
D10 (µm)	1.12 ± 0.08
D50 (µm)	2.45 ± 0.15
D90 (µm)	4.18 ± 0.22
Span	1.25

To elucidate structural interactions and physical state changes during co-spray-drying, FTIR, DSC, and PXRD characterizations were implemented. The core experimental findings are outlined in Table 4. FTIR studies highlighted the conservation of chemical integrity, notably the retention of the Amide I band at 1651 cm⁻¹, validating the secondary structure stability of the KAMP-18 peptide. DSC thermograms displayed distinct melting endotherms for crystalline theophylline and mannitol, while PXRD diffractograms revealed a partial molecular dispersion of theophylline alongside a broad amorphous halo attributed to the peptide matrix.

Table 4. FTIR, DSC and PXRD characterization of the optimized formulation

Technique	Main finding
FTIR	Amide I band retained at 1651 cm ⁻¹ confirming preservation of peptide secondary structure
FTIR	Theophylline carbonyl peaks retained at 1706 and 1660 cm ⁻¹
FTIR	No new absorption bands observed
DSC	Theophylline melting peak at 268.5°C
DSC	Mannitol melting peak at 164.2°C
PXRD	Characteristic β-mannitol peaks retained
PXRD	Reduced theophylline peak intensity indicating partial molecular dispersion
PXRD	Broad amorphous halo attributed to KAMP-18

In Vitro Aerodynamic Dispersion and Ex Vivo Pharmacological Efficacy

The in vitro aerosolization properties of the co-spray-dried powder were evaluated using a Next Generation Impactor (NGI). The results summarized in Table 5 demonstrate outstanding aerodynamic behavior. The emitted dose exceeded 91% for both therapeutic entities. Strikingly, high Fine Particle Fractions (FPF) of $78.42 \pm 2.15\%$ for KAMP-18 and $79.38 \pm 1.88\%$ for Theophylline were achieved. The Mass Median Aerodynamic Diameter (MMAD) fell well within the respirable range (2.78–2.84 μm), combined with narrow geometric standard deviations (GSD), indicating synchronized aerosol co-deposition.

Table 5. In vitro aerodynamic performance of optimized DPI

Parameter	KAMP-18	Theophylline
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Emitted dose (%)	91.42 ± 0.19	93.08 ± 0.31
Fine particle dose (mg)	7.17 ± 0.32	14.78 ± 0.51
Fine particle fraction (%)	78.42 ± 2.15	79.38 ± 1.88
MMAD (μm)	2.84 ± 0.12	2.78 ± 0.10
GSD	1.42 ± 0.05	1.45 ± 0.04

Ex vivo tracheal relaxation parameters were quantified to assess the synergistic bronchodilatory potential of the combination powder. As analyzed in Table 6, the Co-DPI formulation exhibited a remarkably lower EC50 value ($0.66 \pm 0.07 \mu\text{M}$) compared to isolated KAMP-18 ($3.47 \pm 0.45 \mu\text{M}$) and Theophylline ($4.47 \pm 0.52 \mu\text{M}$). A Combination Index (CI) of 0.34 ± 0.05 definitively confirmed strong pharmacological synergism ($\text{CI} < 1$), demonstrating that co-formulation significantly augments tracheal smooth muscle relaxation with lower concentration requirements.

Table 6. Ex vivo tracheal relaxation pharmacological parameters

Parameter	KAMP-18	Theophylline	Co-DPI
EC50 (μM)	3.47 ± 0.45	4.47 ± 0.52	0.66 ± 0.07
Log EC50	-5.46 ± 0.06	-5.35 ± 0.05	-6.18 ± 0.04
E _{max} (%)	96.85 ± 2.45	95.12 ± 2.12	100.0
Hill slope	1.18 ± 0.09	1.26 ± 0.11	1.45 ± 0.12
Combination Index	—	—	0.34 ± 0.05

Biological, Antimicrobial, and Cytocompatibility Profiling

Given that the DPI formulation is targeted for respiratory infections and inflammatory diseases, antimicrobial and antibiofilm activities were determined against key respiratory pathogens. Table 7 indicates that the formulation possessed potent Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC) against *Pseudomonas aeruginosa*, *Klebsiella*

pneumoniae, and MRSA. Notable zones of inhibition (ZOI) and substantial biofilm biomass reductions (e.g., $88.4 \pm 3.2\%$ for *P. aeruginosa*) reflect the formulation's dual anti-infective capacity.

Table 7. Antimicrobial and antibiofilm activities

Organism	MIC ($\mu\text{g/mL}$)	MBC ($\mu\text{g/mL}$)	ZOI (mm)	MBEC ($\mu\text{g/mL}$)	Biomass reduction (%)
<i>P. aeruginosa</i>	2	4	22.1 ± 0.9	6	88.4 ± 3.2
<i>K. pneumoniae</i>	4	8	18.9 ± 0.7	—	—
MRSA	8	16	16.5 ± 0.6	24	76.2 ± 4.5

Cell compatibility and downstream anti-inflammatory activities were assessed using human alveolar (A549) and bronchial (Calu-3) epithelial cells. As shown in Table 8, high cell viabilities ($>92\%$) and high IC₅₀ values highlight the safety margin, validated by a high Selectivity Index of 134. Concurrently, a significant 4.5-fold increase in HDAC2 activity and suppressed levels of pro-inflammatory cytokines (TNF- α , IL-6, and IL-8) illustrate a powerful anti-inflammatory effect.

Table 8. Cell compatibility and anti-inflammatory activity

Parameter	Result
A549 viability (%)	92.8 ± 2.4
Calu-3 viability (%)	93.6 ± 2.1
IC ₅₀ A549 ($\mu\text{g/mL}$)	268.2 ± 10.9
IC ₅₀ Calu-3 ($\mu\text{g/mL}$)	244.1 ± 9.8
Selectivity Index	134
HDAC2 activity	4.5-fold increase

TNF- α (pg/mL)	68.3 $\pm$ 5.1
IL-6 (pg/mL)	85.2 $\pm$ 6.7
IL-8 (pg/mL)	114.6 $\pm$ 9.4

Pharmacokinetic Profiles, Stability, and Safety Margins

In vivo pharmacokinetic evaluation following localized pulmonary delivery demonstrated distinct behaviors for the two actives. As interpreted from Table 9, KAMP-18 displayed prolonged pulmonary residence (>12 h) and minimal systemic exposure, indicating targeted retention in bronchoalveolar lavage fluid (BALF) above MIC levels. Conversely, Theophylline showed rapid systemic absorption (T_{max} = 1.5 h, $t_{1/2}$ = 4.2 h) establishing therapeutic plasma concentrations required for systemic bronchodilation.

Table 9. Pharmacokinetic parameters after pulmonary administration

Parameter	KAMP-18	Theophylline
Pulmonary residence	>12 h	—
T_{max} (h)	—	1.5
Half-life (h)	Minimal systemic exposure	4.2 $\pm$ 0.4
C_{max}	Localized in BALF	Rapid systemic absorption
Therapeutic concentration	Maintained above MIC for 12 h	Within therapeutic plasma range

Finally, Table 10 summarizes the long-term stability and safety evaluations of the optimized DPI. After 6 months of storage under standard and accelerated conditions, the drug content was maintained (>95–97%), and aerosolization metrics (FPF and MMAD) remained statistically unchanged. Hemolysis values remained well below the critical 5% toxicity threshold even at 500 μ g/mL. Histopathological scoring revealed a perfectly normal lung architecture. Notably, the enzymatic half-life of KAMP-18 was extended from 60 min (free peptide) to 180 min within the formulation, alongside significantly enhanced mucus penetration capacity.

Table 10. Stability and safety evaluation

Parameter	Result
Drug content after 6 months (25°C)	>97%
Drug content after accelerated storage	>95%
FPF after storage (%)	77.6 ± 2.1
MMAD after storage (μm)	2.86 ± 0.13
Hemolysis at 100 μg/mL (%)	0.8 ± 0.1
Hemolysis at 500 μg/mL (%)	2.1 ± 0.3
Histopathology	Normal lung architecture
Enzymatic half-life of KAMP-18 (min)	180
Free peptide half-life (min)	60
Mucus penetration	Significantly enhanced compared with free peptide

The response surface optimization confirmed that inlet temperature, feed flow rate, and L-leucine concentration collectively influenced production yield, particle size, and aerosol performance, leading to the selection of the optimized co-spray-dried formulation (Figure 1, Table 1).

Comprehensive physicochemical characterization demonstrated excellent compatibility among KAMP-18, theophylline, and the selected excipients. FTIR spectroscopy confirmed preservation of the peptide secondary structure and theophylline functional groups, while DSC and PXRD analyses verified the coexistence of crystalline and amorphous domains without evidence of chemical degradation or undesirable phase transformation (Figure 2A–C, Table 2).

Morphological evaluation revealed uniformly spherical microparticles with highly corrugated surfaces, moderate positive surface charge, and appropriate geometric characteristics. These properties are known to reduce interparticle cohesion and improve powder dispersibility during inhalation (Figure 3, Table 3).

Aerodynamic characterization demonstrated excellent emitted dose, high fine particle fraction, optimal mass median aerodynamic diameter, and predominant deposition within the bronchiolar and alveolar regions, confirming the suitability of the optimized formulation for efficient pulmonary drug delivery (Figure 4, Table 4).

Ex vivo pharmacological evaluation showed that the reconstituted co-spray-dried formulation produced a marked leftward shift in the concentration-response curve together with a significantly reduced EC_{50} value and complete maximal relaxation, indicating preservation of biological activity and synergistic bronchodilatory efficacy following spray drying (Figure 5, Table 5).

Microbiological investigations confirmed that the antibacterial activity of KAMP-18 was fully retained after formulation. Comparable MIC and MBC values, together with rapid bactericidal kinetics and absence of antagonism in checkerboard analyses, demonstrated that co-delivery with theophylline did not compromise antimicrobial efficacy (Figure 6, Table 6).

The optimized formulation also exhibited potent antibiofilm activity against mature biofilms, producing substantial biomass reduction and low MBEC values, thereby demonstrating efficient penetration and disruption of established biofilm architecture (Figure 7, Table 7).

Cell compatibility studies confirmed excellent pulmonary safety. High epithelial cell viability, elevated IC_{50} values, preservation of normal cellular morphology, and predominantly viable cells following live/dead staining collectively demonstrated excellent cytocompatibility within therapeutically relevant concentration ranges (Figure 8, Table 8).

Mechanistic investigations further demonstrated restoration of HDAC2 activity, suppression of intracellular oxidative stress, and marked downregulation of TNF- α , IL-6, and IL-8 production, supporting the proposed dual antimicrobial and host-directed anti-inflammatory mechanism of the co-delivered formulation (Figure 9, Table 9).

Finally, pharmacokinetic analysis demonstrated prolonged pulmonary retention of KAMP-18, while stability studies confirmed sustained physicochemical integrity and aerodynamic performance during storage. Histopathological examination revealed excellent pulmonary tolerability, whereas hemocompatibility and mucus penetration studies further supported the translational potential of the optimized dry powder inhaler for respiratory drug delivery (Figures 10–13, Table 10).

4. DISCUSSION

4.1 Molecular modeling and formulation rationale

The present study integrated computational modeling, pharmaceutical engineering, and biological evaluation to develop a co-spray-dried dry powder inhaler (DPI) containing the antimicrobial peptide KAMP-18 and the bronchodilator/anti-inflammatory agent theophylline.

This dual-drug strategy was designed to simultaneously eradicate respiratory pathogens while alleviating airway inflammation and bronchoconstriction. Unlike conventional DPI formulations that deliver either antibiotics or bronchodilators alone, the current formulation combines complementary therapeutic mechanisms within a single respirable microparticle, potentially improving treatment efficacy while reducing dosing frequency.

The molecular docking and molecular dynamics simulations demonstrated excellent stability of both active compounds with their respective biological targets (Figure 1). KAMP-18 established a highly stable interaction with lipopolysaccharide (LPS), whereas theophylline maintained stable binding within the HDAC2 catalytic pocket throughout the 200 ns simulation. The backbone RMSD values remained below 0.25 nm for both systems, indicating structural equilibration without major conformational perturbations (Table 1). Furthermore, MM/GBSA calculations revealed substantially stronger binding of KAMP-18 toward LPS than theophylline toward HDAC2, reflecting the electrostatic nature of antimicrobial peptide interactions with bacterial membranes.

These computational findings agree well with recent molecular simulation studies of antimicrobial peptides, which consistently demonstrate that electrostatic attraction between positively charged lysine- and arginine-rich peptides and negatively charged bacterial membranes constitutes the dominant driving force for bacterial membrane disruption. Likewise, recent investigations of low-dose theophylline have confirmed that HDAC2 represents one of its major intracellular therapeutic targets responsible for restoring corticosteroid sensitivity and suppressing chronic airway inflammation.

Unlike previous computational studies that investigated either antimicrobial peptides or bronchodilators independently, the present work simultaneously evaluated both therapeutic agents using molecular docking followed by long-timescale molecular dynamics simulations, thereby providing mechanistic evidence supporting the proposed dual-action therapeutic concept.

4.2 Formulation optimization using Box–Behnken design

The Box–Behnken experimental design successfully optimized the spray-drying process by simultaneously maximizing production yield and aerosol performance while minimizing particle size. The quadratic statistical models demonstrated excellent predictive capability, with coefficients of determination exceeding 0.98 for all critical quality attributes (Table 2), indicating that the selected process variables adequately explained formulation variability.

Among the investigated variables, inlet temperature exerted the greatest positive influence on production yield, whereas feed flow rate primarily controlled particle size. Increasing L-leucine concentration significantly enhanced aerosol dispersion and increased fine particle fraction through modification of particle surface morphology. The response surface analysis suggested that intermediate processing conditions generated the most favorable balance between drying efficiency and particle engineering.

The optimized formulation exhibited a production yield exceeding 72%, encapsulation efficiencies approaching 100% for both active ingredients, and minimal residual moisture (Table

3). These findings indicate that spray drying produced highly efficient drug entrapment with negligible processing losses. Such high encapsulation efficiencies are expected because both KAMP-18 and theophylline remained molecularly dispersed within the homogeneous feed solution prior to atomization.

Recent investigations on spray-dried inhalable peptide formulations similarly report encapsulation efficiencies above 95% when complete solubilization of the therapeutic peptide is achieved before spray drying. Likewise, several recent studies evaluating leucine-containing inhalable powders have demonstrated that increasing leucine concentration improves production yield and aerosolization by promoting rapid surface enrichment during solvent evaporation.

Compared with previously reported peptide DPIs, the present formulation achieved comparable encapsulation efficiency while incorporating two pharmacologically distinct active compounds within a single microparticle system, representing an important advancement over single-drug inhalation platforms.

4.3 Powder engineering and micromeritic characteristics

Particle engineering remains one of the most critical determinants of DPI performance because particle size, density, morphology, and surface chemistry collectively influence aerosol dispersion and pulmonary deposition. The optimized formulation produced particles with a median diameter (D50) of approximately 2.45 μm , narrow size distribution (Span = 1.25), and low residual moisture content (Table 3), all of which fall within the optimal aerodynamic range for deep lung deposition.

Although Carr's Compressibility Index and Hausner Ratio classified the powder as cohesive, these observations should not be interpreted as unfavorable. Highly respirable microparticles commonly exhibit relatively poor bulk flow owing to strong interparticle cohesive forces generated by their extremely small size. However, during aerosolization these cohesive interactions are effectively disrupted by the turbulent airflow produced within the inhaler device.

This phenomenon explains why the present formulation achieved excellent aerodynamic performance despite its relatively high compressibility index. Similar findings have been reported in recent DPI studies, where powders displaying poor static flow nevertheless exhibited outstanding emitted dose and fine particle fraction following aerosolization.

The inclusion of L-leucine played a particularly important role in improving powder performance. During spray drying, leucine rapidly migrated toward the droplet surface because of its limited aqueous solubility, forming a crystalline shell that generated highly corrugated particles with reduced contact area. Consequently, van der Waals attractive forces between neighboring particles decreased substantially, facilitating particle deagglomeration during inhalation.

Recent pharmaceutical engineering studies consistently identify leucine as one of the most effective dispersibility enhancers for inhalable formulations because of its ability to reduce particle cohesion while preserving chemical stability. The present findings strongly support

these observations and further demonstrate that leucine remains equally effective in dual-drug peptide-containing formulations.

4.4 Physicochemical characterization and solid-state properties

Comprehensive physicochemical characterization confirmed that the spray-drying process preserved the structural integrity of both therapeutic agents while producing a physically stable inhalable powder (Table 4). FTIR spectroscopy demonstrated preservation of the characteristic amide I band of KAMP-18 together with retention of the principal carbonyl stretching frequencies of theophylline. Importantly, no new absorption bands appeared following spray drying, indicating absence of chemical interaction or degradation between the peptide, theophylline, and excipients.

DSC analysis similarly confirmed retention of the characteristic melting transitions of mannitol and theophylline, although slight melting point depression was observed within the optimized formulation. Such minor thermal shifts are commonly attributed to dilution effects and molecular dispersion rather than chemical incompatibility. PXRD analysis further demonstrated preservation of crystalline β -mannitol together with partially crystalline theophylline and an amorphous peptide phase.

These complementary analytical techniques collectively indicate that spray drying generated a hybrid solid-state system consisting of crystalline carrier materials embedding molecularly dispersed KAMP-18 without inducing peptide denaturation.

Recent investigations into spray-dried antimicrobial peptides have similarly demonstrated that maintenance of peptide secondary structure following atomization is essential for preserving antimicrobial activity. Furthermore, preservation of partial crystallinity within inhalable formulations has been associated with improved storage stability compared with fully amorphous powders.

The SEM analysis additionally demonstrated highly corrugated spherical particles possessing a rugosity index approaching 1.9, consistent with rapid surface precipitation of leucine during solvent evaporation. Such rough particle surfaces substantially reduce interparticle contact area, thereby enhancing aerosol dispersion. Similar corrugated morphologies have recently been identified as characteristic features of high-performance inhalable powders prepared using leucine-assisted spray drying.

Collectively, the physicochemical characterization demonstrates that the optimized formulation successfully combines excellent structural stability, favorable particle engineering, and appropriate solid-state properties, thereby providing a robust platform for efficient pulmonary drug delivery. These findings, together with the optimization results (Tables 1–4) and aerodynamic evaluation (Table 5), establish a strong physicochemical foundation for the subsequent biological performance discussed in the following sections.

4.5 Preservation of pharmacological activity following co-spray drying

One of the principal concerns associated with spray drying peptide-based therapeutics is the potential loss of biological activity resulting from thermal stress, atomization-induced shear forces, or dehydration. In the present study, the ex vivo tracheal relaxation assay demonstrated that co-spray drying successfully preserved the pharmacological activities of both KAMP-18 and theophylline, while simultaneously producing a formulation with significantly greater bronchodilatory efficacy than either component administered individually (Figure 4, Table 6).

The optimized Co-DPI exhibited an EC_{50} of $0.66 \pm 0.07 \mu\text{M}$, representing approximately a five- to seven-fold improvement in potency compared with the individual drugs. Moreover, maximal relaxation approached 100%, indicating complete preservation of biological activity after formulation processing. The observed leftward shift of the concentration-response curve suggests that co-delivery of both agents enhances pharmacological efficiency rather than merely maintaining their individual effects.

This enhanced activity is likely attributable to complementary mechanisms of action. Theophylline increases intracellular cyclic AMP through phosphodiesterase inhibition while simultaneously restoring HDAC2 activity, resulting in smooth muscle relaxation and attenuation of inflammatory signaling. In contrast, KAMP-18 may modulate epithelial membrane integrity, reduce bacterial burden, and decrease inflammatory mediators that contribute to airway hyperresponsiveness. Together, these mechanisms provide simultaneous bronchodilatory and antimicrobial benefits.

Recent investigations evaluating inhaled peptide therapeutics have similarly demonstrated that appropriate spray-drying conditions preserve peptide bioactivity, while co-formulation strategies have been reported to improve pulmonary therapeutic outcomes by combining complementary mechanisms. Nevertheless, few previous studies have demonstrated the degree of pharmacological enhancement observed in the present work, highlighting the potential of dual-drug inhalable systems.

Importantly, the calculated Combination Index of 0.34 indicates strong pharmacological synergy rather than additive activity (Table 6), supporting the rationale for simultaneous pulmonary delivery of KAMP-18 and theophylline.

4.6 Preservation of antimicrobial activity following formulation

An equally important finding of this study was that spray drying did not compromise the intrinsic antibacterial activity of KAMP-18. Minimum inhibitory concentrations and minimum bactericidal concentrations against *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and methicillin-resistant *Staphylococcus aureus* remained identical before and after formulation, confirming complete preservation of peptide antimicrobial function (Figure 5, Table 7).

The rapid bactericidal activity observed during time-kill studies further supports the membrane-disruptive mechanism predicted by molecular dynamics simulations. Complete bacterial eradication within two hours at four times the MIC is characteristic of membrane-active

antimicrobial peptides and differs fundamentally from conventional antibiotics that inhibit intracellular metabolic targets.

The lack of antibacterial activity exhibited by theophylline was expected and agrees with its established pharmacological profile. More importantly, checkerboard analysis demonstrated a fractional inhibitory concentration index (FICI) of 1.0, indicating that incorporation of theophylline neither enhanced nor inhibited KAMP-18 antimicrobial activity.

This observation has important clinical implications because combination therapies occasionally exhibit antagonistic interactions that reduce antibacterial efficacy. The complete absence of antagonism indicates that simultaneous pulmonary delivery of both agents can be achieved without compromising bacterial killing.

Recent reports investigating inhalable antimicrobial peptides have similarly demonstrated excellent preservation of peptide activity following spray drying, particularly when protective excipients such as mannitol and leucine are employed. However, relatively few studies have evaluated dual-drug inhalable systems combining antimicrobial peptides with bronchodilators, making the present findings particularly relevant for respiratory infections accompanied by airway inflammation.

The retained antimicrobial potency further corroborates the FTIR, DSC, and PXRD analyses (Table 4), which demonstrated preservation of peptide structural integrity after spray drying.

4.7 Antibiofilm efficacy

Biofilm-associated respiratory infections remain among the greatest therapeutic challenges in pulmonary medicine because the extracellular polymeric matrix substantially reduces antibiotic penetration while promoting bacterial persistence. Consequently, demonstrating antibiofilm activity represents an important translational advantage for inhaled antimicrobial therapies.

The present formulation exhibited excellent activity against mature biofilms of *P. aeruginosa* and MRSA (Figure 6, Table 7). Biomass reductions approaching 90% for *P. aeruginosa* and over 75% for MRSA indicate effective penetration of the extracellular polymeric substance and disruption of established bacterial communities.

These findings are consistent with the physicochemical properties of cationic antimicrobial peptides. Electrostatic attraction between positively charged lysine and arginine residues and negatively charged biofilm components facilitates peptide diffusion throughout the biofilm matrix before membrane disruption occurs.

Recent investigations have demonstrated that antimicrobial peptides generally outperform many conventional antibiotics against mature biofilms because their mechanism relies primarily on membrane disruption rather than intracellular metabolic inhibition. Furthermore, pulmonary delivery provides exceptionally high local drug concentrations that cannot readily be achieved through systemic administration.

The relatively low MBEC values observed in the present study therefore support the potential clinical application of the formulation for chronic respiratory infections characterized by

persistent biofilm formation, including cystic fibrosis-associated *P. aeruginosa* infections and chronic obstructive pulmonary disease exacerbations.

4.8 Cytocompatibility and pulmonary safety

A major limitation associated with many membrane-active antimicrobial peptides is their potential toxicity toward mammalian cells. Therefore, demonstrating selective bacterial toxicity while preserving pulmonary epithelial viability represents a critical requirement for clinical translation.

The optimized formulation maintained greater than 90% viability in both A549 and Calu-3 cells at therapeutically relevant concentrations (Figure 7, Table 8). Even at substantially higher concentrations, cell viability remained above 80%, indicating excellent cytocompatibility.

The calculated selectivity index of approximately 134 greatly exceeds the minimum value generally considered acceptable for antimicrobial development. Such a large therapeutic window indicates that bacterial killing occurs at concentrations far below those associated with mammalian cytotoxicity.

Fluorescence microscopy further confirmed preservation of epithelial morphology following treatment. Cells retained their characteristic cobblestone appearance with intact intercellular junctions and minimal membrane damage, contrasting sharply with the positive toxicity control.

These observations likely reflect the fundamental differences between bacterial and mammalian membranes. Bacterial membranes possess abundant negatively charged phospholipids that strongly attract cationic peptides, whereas mammalian cell membranes contain predominantly zwitterionic phospholipids and cholesterol, substantially reducing peptide affinity.

Recent studies investigating host-defense peptides have similarly demonstrated that selective membrane composition largely determines peptide specificity, supporting the favorable safety profile observed in the present work.

4.9 Anti-inflammatory mechanisms and HDAC2 restoration

Beyond its antimicrobial efficacy, the present formulation demonstrated significant anti-inflammatory activity through restoration of HDAC2 function (Figure 8, Table 8). HDAC2 is a critical regulator of inflammatory gene transcription whose activity is markedly reduced during chronic airway inflammation due to oxidative stress.

Treatment with the optimized Co-DPI significantly increased HDAC2 expression and catalytic activity, accompanied by marked suppression of TNF- α , IL-6, and IL-8 production. These findings are highly consistent with the established pharmacology of low-dose theophylline, which has increasingly been recognized as an HDAC2 activator rather than solely a phosphodiesterase inhibitor.

The reduction in intracellular reactive oxygen species further suggests interruption of oxidative stress pathways that contribute to persistent pulmonary inflammation. Because oxidative stress

promotes HDAC2 degradation, attenuation of reactive oxygen species may create a positive feedback mechanism that further enhances anti-inflammatory responses.

An additional advantage of the present formulation lies in its dual therapeutic mechanism. While KAMP-18 rapidly eliminates bacterial pathogens and thereby removes pathogen-associated molecular patterns responsible for initiating inflammatory signaling, theophylline simultaneously suppresses host inflammatory responses by restoring HDAC2-mediated transcriptional regulation.

This integrated antimicrobial and anti-inflammatory strategy differs fundamentally from conventional inhaled antibiotics, which eradicate pathogens but exert minimal direct effects on inflammatory signaling. Likewise, bronchodilators alone cannot eliminate persistent bacterial infection.

Recent investigations increasingly emphasize that simultaneous control of infection and inflammation is essential for successful treatment of chronic respiratory diseases. The present findings therefore provide compelling evidence that the co-spray-dried DPI achieves this objective through complementary molecular mechanisms.

Collectively, the pharmacological, microbiological, cytocompatibility, and mechanistic findings (Tables 6–8) demonstrate that the optimized formulation successfully preserves the biological activities of both therapeutic agents while providing synergistic bronchodilatory effects, potent antimicrobial and antibiofilm activity, excellent pulmonary safety, and significant suppression of inflammatory signaling. These results establish a strong biological foundation for the in vivo pharmacokinetic, stability, and safety evaluations discussed in the following section.

4.10 In vivo pharmacokinetics and pulmonary drug disposition

The pharmacokinetic evaluation demonstrated that the co-spray-dried DPI successfully achieved the intended dual pharmacokinetic profile, namely prolonged pulmonary retention of KAMP-18 combined with rapid systemic absorption of theophylline (Figure 9, Table 9). Following intratracheal administration, KAMP-18 concentrations in bronchoalveolar lavage fluid remained above the MIC against *P. aeruginosa* for approximately 12 h, whereas theophylline reached its maximum plasma concentration within 1.5 h and exhibited an elimination half-life of approximately 4.2 h. This pharmacokinetic behavior reflects the complementary therapeutic objectives of the formulation, whereby the antimicrobial peptide remains localized within the lung to maximize bacterial eradication while minimizing systemic exposure, whereas theophylline rapidly enters the systemic circulation to provide early bronchodilation and anti-inflammatory activity.

The prolonged pulmonary residence of KAMP-18 can be attributed to several formulation characteristics. First, the optimized aerodynamic diameter (approximately 2.8 μm) promoted efficient alveolar deposition. Second, the moderately positive surface charge facilitated electrostatic interaction with negatively charged airway mucus and epithelial surfaces. Third, the spray-dried mannitol/leucine matrix provided gradual dissolution following deposition, thereby delaying peptide clearance. These observations agree with recent investigations demonstrating that inhalable peptide formulations exhibiting aerodynamic diameters between 1 and 3 μm show

significantly prolonged pulmonary retention compared with larger particles (15). Likewise, recent pharmacokinetic studies of inhaled antimicrobial peptides have reported that local pulmonary delivery markedly reduces systemic exposure while maintaining therapeutic concentrations within the respiratory tract (16).

Compared with conventional oral or intravenous administration of theophylline, pulmonary delivery offers several pharmacokinetic advantages, including lower systemic doses, reduced plasma fluctuations, and decreased risk of dose-related adverse effects. Consequently, the present formulation may improve the therapeutic index of theophylline while simultaneously providing sustained antimicrobial protection through localized peptide delivery.

4.11 Solid-state stability and long-term formulation robustness

Long-term physicochemical stability is essential for successful translation of inhalable peptide formulations because moisture uptake, particle agglomeration, and peptide degradation can substantially compromise aerosol performance. In the present study, the optimized DPI maintained excellent chemical and physical stability under both long-term and accelerated storage conditions (Figure 10, Table 10). HPLC analysis demonstrated that both KAMP-18 and theophylline retained more than 97% chemical integrity during long-term storage and more than 95% under accelerated conditions, while no significant degradation products were detected.

The preserved aerodynamic performance after six months further confirmed the robustness of the optimized formulation. Fine particle fraction remained essentially unchanged, whereas MMAD remained within the optimal range for deep lung deposition. These observations indicate that the spray-dried particles resisted moisture-induced aggregation despite prolonged storage under elevated temperature and humidity.

The excellent storage stability is likely attributable to the complementary functions of the excipients. Mannitol provided a physically stable crystalline matrix with low hygroscopicity, while leucine formed a hydrophobic surface shell that reduced moisture adsorption. This interpretation is supported by the FTIR, DSC, and PXRD analyses (Table 4), which confirmed preservation of both peptide structure and crystalline carrier phases throughout the study.

Recent investigations on inhalable peptide powders similarly report that crystalline mannitol and leucine substantially improve storage stability by minimizing moisture-induced particle fusion and preserving aerosolization efficiency (17). Therefore, the present findings further support the growing consensus that leucine-assisted spray drying represents one of the most effective approaches for stabilizing inhalable peptide therapeutics.

4.12 Pulmonary tolerability and systemic safety

An equally important objective of the present investigation was to establish the pulmonary safety of repeated administration. Histopathological examination demonstrated that daily administration of the optimized formulation for 14 consecutive days produced no detectable structural abnormalities within lung tissues (Figure 11, Table 10). The alveolar architecture remained intact, bronchial epithelial morphology was preserved, and no evidence of inflammatory infiltration, edema, hemorrhage, or fibrotic remodeling was observed.

These observations are particularly important because membrane-active antimicrobial peptides may potentially damage host tissues if insufficient selectivity is achieved. However, the absence of pulmonary toxicity observed in the present study confirms that KAMP-18 selectively targets bacterial membranes without disrupting mammalian pulmonary cells, consistent with the cytocompatibility results discussed previously (Table 8).

The hemocompatibility assay further demonstrated excellent systemic safety. Hemolysis remained below 1% at therapeutically relevant concentrations and below 3% even at supratherapeutic doses, values substantially below the internationally accepted 5% threshold for hemocompatible biomaterials (Table 10). Such findings indicate that accidental systemic exposure is unlikely to produce clinically relevant erythrocyte damage.

Recent inhalation toxicology studies have similarly concluded that well-designed inhalable peptide formulations exhibit excellent pulmonary tolerability when formulated with biocompatible excipients such as mannitol and leucine (18). The present findings therefore strengthen the safety profile of the proposed formulation and support its potential progression toward advanced preclinical evaluation.

4.13 Enzymatic protection and mucus penetration

One of the principal limitations of therapeutic peptides is their rapid degradation by endogenous proteases present within the respiratory tract. The present formulation successfully addressed this limitation by significantly increasing peptide stability in simulated lung fluid. KAMP-18 encapsulated within the spray-dried matrix exhibited a three-fold longer enzymatic half-life than the free peptide (Figure 12, Table 10), indicating substantial protection against proteolytic degradation.

This protective effect is likely attributable to gradual matrix dissolution, which delays peptide exposure to extracellular proteases while simultaneously maintaining sustained local drug concentrations. Moreover, the optimized microparticles demonstrated efficient diffusion across simulated pulmonary mucus, suggesting that the formulation can effectively penetrate mucus barriers before releasing the peptide into infected tissues.

Recent pulmonary drug delivery studies increasingly emphasize that successful respiratory peptide formulations must overcome both enzymatic degradation and mucus entrapment. The current findings therefore represent an important translational advantage, particularly for chronic respiratory infections characterized by excessive mucus production, including cystic fibrosis and advanced chronic obstructive pulmonary disease (16,17).

4.14 Overall aerosolization performance

The aerodynamic performance of the optimized formulation represents one of its most significant strengths. The synchronized deposition profiles of KAMP-18 and theophylline across the NGI stages confirmed that both active ingredients remained uniformly incorporated within the same respirable microparticle population (Figure 13, Table 5). The emitted dose exceeded 90% for both compounds, while fine particle fractions approached 80%, values substantially higher than those reported for many conventional DPI formulations.

The MMAD values of approximately 2.8 μm indicate highly efficient deposition within the bronchiolar and alveolar regions, thereby maximizing therapeutic exposure at the primary sites of respiratory infection and inflammation. Furthermore, the relatively narrow geometric standard deviation suggests reproducible aerosol generation and predictable pulmonary deposition.

These findings can largely be attributed to the optimized particle engineering achieved through Box–Behnken optimization (Tables 1–3). Specifically, the corrugated surface morphology generated by leucine reduced interparticle cohesion while preserving particle integrity during aerosolization. Similar observations have been reported in recent investigations of leucine-containing inhalable powders, where corrugated particles consistently demonstrate superior dispersibility and deep-lung delivery compared with smooth spherical particles (17,18).

4.15 Overall significance, strengths, limitations, and future perspectives

Collectively, the present study demonstrates the successful development of a rationally engineered co-spray-dried DPI capable of simultaneously delivering an antimicrobial peptide and a bronchodilator/anti-inflammatory agent. Integration of computational modeling, formulation optimization, physicochemical characterization, microbiological evaluation, pharmacological assessment, pharmacokinetic analysis, and safety studies provides comprehensive evidence supporting the feasibility of this dual-drug pulmonary delivery strategy.

Several strengths distinguish this work from previously published inhalable peptide formulations. First, molecular docking and molecular dynamics simulations provided mechanistic support for both therapeutic components before formulation development. Second, statistical optimization using a Box–Behnken experimental design enabled systematic identification of the optimal processing conditions. Third, the formulation demonstrated preservation of antimicrobial activity, enhanced bronchodilatory efficacy, potent antibiofilm activity, excellent cytocompatibility, favorable pharmacokinetics, prolonged peptide stability, and outstanding aerosol performance within a single inhalable platform. Collectively, these findings suggest that simultaneous pulmonary delivery of KAMP-18 and theophylline may provide a promising therapeutic strategy for respiratory infections complicated by airway inflammation.

Despite these encouraging findings, several limitations should be acknowledged. The pharmacokinetic and safety studies were performed in healthy experimental animals rather than infected disease models. Moreover, although ex vivo and in vitro experiments demonstrated strong therapeutic activity, future investigations should evaluate long-term efficacy in clinically relevant models of bacterial pneumonia or chronic pulmonary infection. Additional studies examining large-scale manufacturing, device compatibility, and extended storage stability beyond six months would further facilitate clinical translation.

Overall, the present work provides a strong preclinical foundation for the development of multifunctional inhalable peptide formulations. The combination of localized antimicrobial therapy with simultaneous bronchodilatory and anti-inflammatory effects represents an attractive strategy that may improve treatment outcomes for chronic respiratory diseases while reducing systemic toxicity and minimizing antimicrobial resistance. Future clinical investigations are warranted to confirm the translational potential of this novel co-spray-dried dry powder inhaler.

CONCLUSION

The present study successfully developed and comprehensively characterized a novel co-spray-dried dry powder inhaler (DPI) incorporating the antimicrobial peptide KAMP-18 and theophylline for pulmonary delivery. Molecular docking and molecular dynamics simulations confirmed stable molecular interactions, demonstrating strong electrostatic binding of KAMP-18 to bacterial lipopolysaccharide and stable occupancy of theophylline within the HDAC2 catalytic pocket. These computational findings supported the proposed dual antibacterial and anti-inflammatory therapeutic mechanism.

Optimization using a Box–Behnken experimental design produced highly respirable microparticles with excellent production yield, high encapsulation efficiency, narrow particle-size distribution, corrugated particle morphology, and favorable physicochemical characteristics. FTIR, DSC, PXRD, SEM, and zeta potential analyses demonstrated excellent compatibility between formulation components while preserving the structural integrity of both active compounds throughout the spray-drying process.

The optimized formulation exhibited outstanding aerodynamic performance, with high emitted dose, fine particle fraction approaching 80%, and an MMAD within the optimal range for deep lung deposition. These characteristics translated into efficient pulmonary delivery while minimizing oropharyngeal deposition.

Importantly, the formulation completely preserved the biological activity of KAMP-18 following spray drying. Potent antibacterial activity against *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and methicillin-resistant *Staphylococcus aureus* (MRSA) was maintained without antagonistic interaction from the co-delivered theophylline. Furthermore, the formulation demonstrated remarkable antibiofilm efficacy against mature bacterial biofilms, highlighting its potential for managing chronic pulmonary infections associated with biofilm formation.

Ex vivo pharmacological evaluation revealed significant synergistic bronchodilatory activity, with the co-formulation producing markedly greater tracheal relaxation than either individual component alone. Mechanistic investigations further demonstrated restoration of HDAC2 activity, suppression of oxidative stress, and substantial reduction of pro-inflammatory cytokine production, supporting the therapeutic rationale for combining antimicrobial and anti-inflammatory activities within a single inhalable formulation.

The optimized DPI also demonstrated excellent pulmonary biocompatibility, minimal cytotoxicity toward respiratory epithelial cells, favorable hemocompatibility, enhanced enzymatic stability, efficient mucus penetration, prolonged pulmonary residence of KAMP-18, and acceptable systemic pharmacokinetics of theophylline. Long-term stability studies confirmed preservation of both chemical integrity and aerodynamic performance under recommended storage conditions.

Overall, this multifunctional co-spray-dried DPI represents a promising inhaled therapeutic platform capable of simultaneously eradicating bacterial pathogens, disrupting biofilms, reducing airway inflammation, and improving pulmonary function. These findings support further

preclinical efficacy investigations and subsequent clinical translation for the treatment of chronic bacterial respiratory diseases, including difficult-to-treat pulmonary infections characterized by persistent inflammation and antimicrobial resistance.

Abbreviations

Abbreviation	Definition
AMP	Antimicrobial Peptide
ANOVA	Analysis of Variance
BALF	Bronchoalveolar Lavage Fluid
BBD	Box–Behnken Design
CI	Combination Index
CI	Carr's Compressibility Index (when referring to powder flow)
Co-DPI	Co-spray-dried Dry Powder Inhaler
DSC	Differential Scanning Calorimetry
DLS	Dynamic Light Scattering
DPI	Dry Powder Inhaler
ED	Emitted Dose
EE	Encapsulation Efficiency
ELISA	Enzyme-Linked Immunosorbent Assay
EPS	Extracellular Polymeric Substance
FICI	Fractional Inhibitory Concentration Index
FPD	Fine Particle Dose
FPF	Fine Particle Fraction
FTIR	Fourier Transform Infrared Spectroscopy
GSD	Geometric Standard Deviation
GSH	Glutathione
H&E	Hematoxylin and Eosin
HDAC2	Histone Deacetylase 2
HPLC	High-Performance Liquid Chromatography
HR	Hausner Ratio

IC ₅₀	Half-Maximal Inhibitory Concentration
IL	Interleukin
KAMP-18	Keratin-Derived Antimicrobial Peptide-18
LPS	Lipopolysaccharide
MBEC	Minimum Biofilm Eradication Concentration
MBC	Minimum Bactericidal Concentration
MD	Molecular Dynamics
MIC	Minimum Inhibitory Concentration
MMAD	Mass Median Aerodynamic Diameter
MM/GBSA	Molecular Mechanics/Generalized Born Surface Area
MM/PBSA	Molecular Mechanics/Poisson–Boltzmann Surface Area
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide
NF-κB	Nuclear Factor Kappa B
NGI	Next Generation Impactor
PBS	Phosphate Buffered Saline
PDE	Phosphodiesterase
PK	Pharmacokinetics
PXRD	Powder X-ray Diffraction
ROS	Reactive Oxygen Species
RMSD	Root Mean Square Deviation
RMSF	Root Mean Square Fluctuation
RT-qPCR	Reverse Transcription Quantitative Polymerase Chain Reaction
SEM	Scanning Electron Microscopy
SI	Selectivity Index
SOD	Superoxide Dismutase
USP	United States Pharmacopeia
ZOI	Zone of Inhibition

Declarations

****Abbreviations****

BBD: Box-Behnken Design; CLSM: Confocal Laser Scanning Microscopy; DPI: Dry Powder Inhaler; DSC: Differential Scanning Calorimetry; FPF: Fine Particle Fraction; FTIR: Fourier-Transform Infrared Spectroscopy; HDAC2: Histone Deacetylase 2; KAMP-18: Keratinocyte Antimicrobial Peptide 18; MBC: Minimum Bactericidal Concentration; MBEC: Minimum Biofilm Eradication Concentration; MBIC: Minimum Biofilm Inhibitory Concentration; MIC: Minimum Inhibitory Concentration; MMAD: Mass Median Aerodynamic Diameter; NGI: Next Generation Impactor; PXRD: Powder X-Ray Diffraction; ROS: Reactive Oxygen Species; SEM: Scanning Electron Microscopy.

****Ethics approval and consent to participate****

All experimental animal protocols were approved by the Institutional Animal Care and Use Committee (CU-IACUC), Faculty of Pharmacy, Cairo University (Approval No. CU-IACUC-2026-M42). Human blood samples for hemocompatibility assays were obtained following donor consent under the same institutional protocol approval.

****Consent for publication****

Not applicable.

****Availability of data and materials****

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

****Competing Interests****

The author declares that there are no competing financial or non-financial interests.

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****Authors' contributions****

M.M.S.K. performed conceptualization, experimental design, molecular modeling, formulation development, aerosol characterization, in vitro/ex vivo biological evaluations, data analysis, manuscript drafting, and final revision.

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