Spray-drying of inhalable, multifunctional formulations for the treatment of biofilms formed in cystic fibrosis

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Abstract:

Cystic fibrosis (CF) is a serious lung disease, commonly susceptible to *Pseudomonas aeruginosa* colonization. The dense mucus together with biofilm formation limit drug permeability and prevent the drug from reaching the site of action, causing treatment failure of the bacterial infection. Besides the use of antibiotics, the mucolytic agent *N*-acetylcysteine (NAC) is recommended to be co-administered in the treatment of CF. Although several formulations have been developed for inhalation therapy to improve the pulmonary condition in CF patients, there is still no comprehensive study on a combined multifunctional dry powder formulation of antibiotics with NAC. In this work, we developed an innovative multifunctional dry powder inhaler (DPI) formulation based on salt formation between NAC and antibiotics and characterized their solid state properties and physical stability. NAC could be spray dried together with three different antibiotics, azithromycin (Azi), tobramycin (Tobra) and ciprofloxacin (Cipro), without the use of organic solvents to form Azi/NAC, Tobra/NAC and Cipro/NAC DPI formulations. Solid-state characterization of these DPI formulations showed that they were amorphous after spray drying. Azi/NAC and Tobra/NAC form co-amorphous salt systems that were physically stable under storage at stress conditions. For particle characterization, the obtained mass median aerodynamic diameters were in a suitable range for inhalation ($< 5.0\ \mu\text{m}$). The multifunctional antibiotic/NAC formulations conserved or improved the antibiotic susceptibility and showed promising results regarding the inhibition of *P. aeruginosa* PA14 biofilm formation.

Keywords: Salt formation, antibiotics, co-amorphous drug delivery, inhalation, bacterial infection, dry-powder formulation

1. Introduction

Cystic fibrosis (CF) is a congenital lethal chronic disease in which thick and sticky mucus is secreted in different organs such as the pancreatic duct, intestine, and the lung [1]. The abnormal thickness of the mucus causes problems such as intestinal blockage and a reduced clearance of the airways. Infections by *Pseudomonas aeruginosa* that built resistant biofilms in the lung are common in the later stages of the disease [2]. According to recent statistics, approximately 70,000 to 100,000 children and adults around the world suffer from CF [3, 4].

The strategies for treating CF in the lung partly rely on the application of inhaled antibiotics in high doses using nebulizers for the treatment of early infections and inhibition of biofilm formation, either as monotherapy or in addition to oral/IV antibiotic administration as combination therapy [5, 6]. The guidelines for treating CF recommend co-administration of a mucolytic agent to fluidize the mucus, as well as of osmotic compounds like mannitol, to preserve hydration of the airways [7, 8]. The key advantage of inhalation of antibiotics is to reduce systemic side-effects by avoiding systemic circulation [9-12]. Currently, only few antibiotics, namely tobramycin (Tobi®, Novartis AG, Switzerland), aztreonam (Cayston®; Gilead Sciences, USA), and colistimethate sodium (Colomycin®, Promixin®) are available on the market for application via nebulization [13-17]. Nebulization, however, has several drawbacks including the potential for the nebulizer itself to act as a source of bacterial infection, drug loss during the aerosolization process, a long administration time, and a reduced performance of the nebulizer over time [18-24]. In contrast to nebulization, the use of dry-powder inhalers (DPIs) reduces treatment expenses and increases patient convenience, and thus therapy adherence. In addition, DPIs do not require cold temperature storage of the formulation, as is the case for nebulizers [25], have a lower contamination probability and allow higher doses to be delivered [26].

N-acetylcysteine (NAC) has antimicrobial properties by decreasing biofilm formation and diminishing the production of extracellular polysaccharide matrix [27-29]. NAC has a mucolytic

effect by cleaving the disulfide bonds in mucins leading to reduced mucus viscosity [28, 30, 31]. Based on the essential need to combine antibiotics and mucolytics for an efficient therapy, the aim of this study was to combine these drugs in one formulation. This combination could thus potentially facilitate the treatment of CF patients. In the present study, we describe the preparation of a spray-dried powder for inhalation composed of antibiotics and NAC.

Spray drying follows the thermodynamic pathway of producing amorphous drugs [32]. Whilst amorphous drugs may still have some residual short-range molecular order [32], they do not have any positional and orientation long-range order (which would reduce the free energy of the material), and are thus physically unstable and can recrystallize. This is because the molecules are mobile, even in their glassy form (due to β -relaxation), *i.e.*, even at temperatures below their glass transition temperature [33]. The physical stability of amorphous drugs, however, can be improved by formulating them as glass solutions or amorphous solid dispersions [34, 35]. Polymeric glass solutions stabilize the amorphous drugs by molecularly incorporating the drug into an amorphous polymeric matrix [34, 35]. Non-polymeric glass solutions (so called co-amorphous systems) stabilize the drug molecules by means of another small molecule or excipient *via* co-amorphization. Co-amorphization is a process that involves amorphizing a crystalline drug together with another also initially crystalline low molecular weight material, the so-called coformer (which can be a second drug or an excipient) [36, 37]. Co-amorphous systems thus contain two components but are characterized by a single glass transition temperature (T_g) [37, 38]. Co-amorphization results in the formation of co-amorphous salts and has an added advantage as it improves the solubility of the drug by two solid-state conversions, namely salt formation and amorphization [39].

In this study, after producing multifunctional DPI formulations, solid-state characterization and physical stability of the obtained multifunctional DPI formulations were investigated as well as their aerodynamic properties and disintegration behaviour. To understand the potential for mucus interaction, the influence of the multifunctional DPI formulation on the viscosity of

mucus was studied. Finally, the activities of the antibiotics on bacteria and their biofilm were tested.

2. Material and methods

2.1. Materials

D-mannitol ($\geq 98\%$) (MW = 182.172 g/mol), *N*-acetylcysteine (MW = 163.19 g/mol, $pK_a=3.82$), L-leucine (MW = 131.18 g/mol), agarose, azithromycin (MW = 748.98 g/mol, $pK_a=12.43$ [40]), ciprofloxacin (MW = 331.346 g/mol, $pK_a=8.68$ [40]) and tobramycin (MW = 467.515 g/mol, $pK_a=12.57$ [40]) were obtained from Sigma Aldrich (Steinheim, Germany). Nitrocellulose membrane was a gift from Fisher Scientific GmbH (Schwelten, Germany). Pulmonary horse mucus was obtained after bronchoalveolar lavage (BAL) of horses (Pferdeklinik Altforweiler, Germany).

Materials for preparation of proteose peptone glucose ammonium salts (PPGAS) medium: ammonium chloride (VWR, Darmstadt Germany), potassium chloride (Grüssing GmbH, Filsum, Germany), Tris-HCl (Carl Roth, Karlsruhe, Germany), anhydrous magnesium sulfate (Grüssing GmbH, Filsum, Germany), tryptone (BD Biosciences), glucose (Carl Roth, Karlsruhe, Germany). Lysogeny broth (LB) medium (Lennox) was purchased from Carl Roth (Karlsruhe, Germany). *P. aeruginosa* PA14 strain was kindly provided by the Häußler Lab (Helmholtz Centre for Infection Research, Germany).

2.2. Preparation of multifunctional DPI formulations

The multifunctional DPI formulations were produced by using a BÜCHI B-290 Spray Dryer (Flawil, Switzerland). NAC and the antibiotics were separately dissolved in Milli-Q water under magnetic stirring. The NAC solution was added slowly to the antibiotics solution at different ratios (Table 1). The mixed solution was left for 10 min until it became clear and L-leucine was added to improve the aerodynamic properties and reduce cohesion of the spray-dried particles [41]. The amount of L-leucine (LEU), given in Table 1, was determined in preliminary studies. The mixed solution was adjusted to gain a total concentration of all solid substances of 1 wt. %

for spray drying. For quantification, 100 μ L of a 5 mg/mL rhodamine B-ethanol solution was added for each 100 mg of dry substance in the feeding solution for spraying. The inlet temperature was 80 °C and the gas flow rate 35 m³/h (aspirator was set to 100%). The air volume flow was 1050 L/h (gas rotameter was set to 50 mm) and the feeding solution had a flow of $\sim$ 3 mL/min. All formulations were spray dried with compressed air. Then, the obtained powders were collected and stored in a desiccator at room temperature. All formulations were spray dried in triplicate. The multifunctional DPI formulations obtained were ciprofloxacin/NAC (Cipro/NAC), azithromycin/NAC (Azi/NAC), tobramycin/NAC (Tobra/NAC).

2.3. Solid-state characterisation

An X-ray powder diffractometer (XRPD) was used to determine the solid-state form of the DPI formulations. The measurements were performed using an X'Pert PRO XRPD (PANalytical, Almelo, The Netherlands). In brief, the crystalline starting materials and DPI formulations were exposed to Cu K α radiation (1.5418 Å) which was generated using a voltage and current of 45 kV and 40 mA, respectively. The samples were scanned in reflectance mode from 0 – 35° 2 θ . Thermal analysis was performed using a modulated differential scanning calorimeter (mDSC) (Discovery, TA Instruments, New Castle, USA). Approximately 4 mg of the crystalline samples and DPI formulations were crimped into a T_{zero} pan and covered with a T_{zero} lid. The experiments were conducted in a modulated temperature mode using a heating rate of 2 °C/min, an amplitude of 0.212 °C and period of 40 s. For the NAP, Tobra, and Azi crystalline samples quench cooling (QC) was performed before determining T_g . Crystalline ciprofloxacin was converted to the amorphous form by ball milling before determining T_g . For the DPI formulations, the samples were analysed from –20 °C to 20 °C above the respective T_g . A theoretical estimate of the T_g of the co-amorphous DPI formulations was made using the Fox equation [42]:

$$\frac{1}{T_{g12}} = \frac{w_1}{T_{g1}} + \frac{w_2}{T_{g2}}$$

Where T_{g12} is the theoretical T_g of the co-amorphous formulation, T_{g1} and T_{g2} are the experimental T_g of the neat amorphous drug and NAC respectively, and w_1 and w_2 are weight fractions of the drug and NAC, respectively.

2.4. Morphology of the multifunctional DPI formulations

For morphology analysis, a scanning electron microscope EVO HD 15 from Zeiss (Jena, Germany) was used. Each of the multifunctional DPI formulations was spread on an individual carbon disc and sputter-coated with a 10 nm gold layer using a Quorum Q150R ES sputter coater (Laughton, UK).

2.5. Determination of in vitro aerodynamic behavior

For examination of the aerodynamic properties, the multifunctional DPI formulations were applied to a Next Generation Impactor (NGI) (Copley Scientific, Nottingham, UK). Before the experiment, the impactor pans were coated with a Brij-coating consisting of 4 parts 15% Brij 35 in ethanol in 6 parts glycerol; 10 mL of Milli-Q water was filled into the pre-separator. For every experiment, a hard gelatin capsule (size 3) was filled with approximately 20 mg formulation. The air flow for application was set to 60 L/min, controlled by a M1A flowmeter (Copley Scientific, Nottingham, UK). The capsules were placed in a HandiHaler (Boehringer Ingelheim, Ingelheim, Germany) and were pierced. Aerosolization of the powder was achieved by applying a 4 second gas flow by a vacuum pump and critical flow controller (both Erweka, Heusenstamm, Germany). Then, the powders deposited in the different NGI cups were quantified by dissolving with a defined amount of water and analyzing the fluorescence signal of rhodamine B using a Tecan reader infinite 200 (Tecan, Männedorf, Switzerland). For each formulation, an individual calibration curve was prepared and the whole formulation was analyzed using an excitation wavelength of 565 nm and an emission wavelength of 625 nm. All experiments of every formulation were carried out in triplicate.

2.6. *Disintegration behavior of multifunctional DPI formulations*

0.1% agarose gels were prepared by dissolving agarose in hot water for 30 min and pouring the solution into Petri dishes. The gel was left overnight to cool down. Spray-dried multifunctional DPI formulations were spread on top of a nitrocellulose membrane using a Penn-Century device (Wyndmoor, USA). Then, the nitrocellulose membrane pieces were placed on top of the gel pad for a pre-determined amount of time (5 min, 10 min, and 30 min) to determine the disintegration behavior of the formulations over time. Afterwards, the membranes were removed from the gel pads and analyzed by SEM. All experiments were carried out in triplicate.

2.7. *Determination of the rheological properties of mucus after applying the multifunctional DPI formulations*

The viscoelastic properties of pulmonary horse mucus in the presence and without the formulations were determined using a Kinexus rotational viscometer (Malvern, Malvern, UK). Pulmonary horse mucus was used as an alternative to human mucus due to its availability in quantities necessary for conducting the experiments. The viscosity of the pulmonary horse mucus (Pferdeklinik Altforweiler, Germany) without treatment with the multifunctional DPI formulations was measured as a control. The effect of pure NAC on the viscosity of mucus was also measured. To investigate the effect of the formulations, every mucus sample (500 μ L) was treated with 100 μ L dissolved formulation adjusting the NAC content to 0.1%. The viscous behaviour was measured using a cone-plate geometry with 0.5° angle (C60/0.5) at different shear stress rates (0.02 – 100 Pa). Measurements were performed at room temperature in triplicate.

2.8. *Stability of the multifunctional DPI formulations*

2.8.1. *Physical stability*

Physical stability (conversion from the amorphous form to the crystalline state) of the multifunctional DPI formulations was analyzed by XRPD after storage of the samples for 6

weeks at 65 °C (*i.e.*, in the glassy state) and for 20 minutes at 125 °C (*i.e.*, in the supercooled liquid state).

2.8.2. Morphological stability determined by SEM

For stability analyses, a scanning electron microscope EVO HD 15 from Zeiss (Jena, Germany) was employed. For each formulation, 2 batches were prepared. One batch was stored in a desiccator in the presence of silica gel at room temperature and one at ambient conditions at room temperature in the dark. These storage conditions were chosen to enable a comparison between formulations that were stored at dry conditions (~0% RH) and at ambient/humid conditions (45% RH). The formulations were investigated regarding their morphological changes directly after preparation, and after two weeks, three months, and nine months of storage.

2.9. *Pseudomonas aeruginosa* biofilm assay

Determination of the effects of the multifunctional DPI formulations on the overall biofilm mass was performed using the crystal violet (CV) assay according to reported procedures with slight modifications [43, 44]. *P. aeruginosa* PA14 was cultivated in 96-well plates using PPGAS medium (0.02 M NH₄Cl, 0.02 M KCl, 0.12 M Tris-HCl, 0.0016 M MgSO₄, 1% Tryptone, 0.5% glucose, pH 7.2). Two µL of the multifunctional DPI formulations stock solution in water were added to 198 µL of the bacterial culture to give a total volume of 200 µL (final concentration equals 1% of the stock solution concentration). Neat antibiotics: azithromycin (0.03 and 1.0 mg/mL in DMSO), ciprofloxacin (0.01 and 0.03 mg/mL in DMSO), tobramycin (0.01, 0.03, and 1.0 mg/mL in water) as well as NAC (aqueous solutions at the same concentrations as in the multifunctional DPI formulations) were used as references. DMSO 1% was used as a control. Experiments were performed in triplicate. Graphical illustrations represent the mean values and error bars denote the standard deviation.

2.10. Extracellular DNA (eDNA) assay

The impact of multifunctional DPI formulations on eDNA was assessed as previously reported [44] [45] via incubation of biofilm with propidium iodide solution (0.05 mg/mL) at 37 °C for 24 h and detection of specific fluorescence at 620 nm after a thorough washing step with Milli-Q water. DMSO 1% was used as a control. Experiments were performed in triplicate. Graphical illustrations of the results represent the mean values and error bars denote the standard deviation.

2.11. Antibacterial activity assay

Determination of growth inhibitory effects and minimum inhibitory concentrations (MICs) for the multifunctional DPI formulations and the antibiotics were performed as described in literature [46] [47]. Briefly, MIC values against *P. aeruginosa* PA14 were determined in 96-well plates (Sarstedt, Nümbrecht, Germany). As bacteria start OD₆₀₀, 0.03 was used in a total volume of 200 µL in LB medium containing the multifunctional DPI formulations/antibiotics dissolved in water or DMSO (maximal DMSO concentration in the experiments was 1%). Six concentrations of the antibiotics (in duplicate) were prepared by two-fold serial dilution starting from 0.1 - 0.3 µg/mL according to their concentration in the multifunctional DPI formulations. The ODs were measured using a PHERAstar Microplate reader (BMG Labtech, Ortenberg, Germany) after inoculation and incubation for 18 h at 37 °C with 200 rpm. Given MIC values are means of two independent determinations and defined as the lowest concentration of compound that reduced OD₆₀₀ by $\geq 95\%$. Percent inhibition of bacterial growth was calculated for azithromycin, tobramycin, and the corresponding multifunctional DPI formulations, where no complete inhibition of bacterial growth was observed at the highest concentration (0.3 µg/mL).

2.12. Statistical analysis

All statistical calculations were performed using GraphPad Prism 8.0.1 software. A one sample *t*-test was performed to calculate the statistical significance of the mean compared to the mean

of the DMSO control (100% biofilm or eDNA formation). An unpaired *t*-test with two-tailed *P* value < 0.05 was used to compare significance between two groups.

3. Results and discussion

3.1. Spray drying NAC with antibiotics

Spray drying of drugs is an attractive approach to obtain micronized particles suitable for inhalation. Working with NAC as a functional matrix for spraying has not been achieved yet [48]. Spraying of pure NAC has resulted in strongly agglomerated particles (Fig. 1) and also the collection of the formulation was complicated due to the hard and sticky layer-like structure formed on the spray dryer's collector glass.

Mixing NAC with a compound offering an opposite charge was assumed to regulate hygroscopicity by salt formation. For complex formation, potentially charge-bearing antibiotics with different numbers of amino groups were chosen. To simultaneously represent different classes of antibiotics, ciprofloxacin (a fluoroquinolone), azithromycin (a macrolide antibiotic), and tobramycin (an aminoglycoside) were selected. For ciprofloxacin only an equimolar amount of NAC was necessary due to the fact that only the secondary amino group in the piperazine heterocycle is an amino group that can be ionized (the other two nitrogen atoms are in a vinylogous amide interaction with the keto groups inside the molecule, and for this reason, vinylogous amides are hard to protonate). To complex azithromycin, a molar ratio of 1:2 was chosen to address the two potential positive charges. For tobramycin, an already water-soluble antibiotic with five amino groups that are easily protonated, a molar ratio of 1:5 of tobramycin to NAC was chosen. These ratios were selected to achieve optimal concentrations for salt formation within the formulation. These ratios should also facilitate the formation of stable formulations of the spray-dried powder balancing the high hygroscopic potential of NAC. The ionization sites of all components are shown in Fig. 2. Salt formation could also improve the solubility necessary for spray drying from aqueous solution; *e.g.*, dissolving azithromycin and NAC using a molar ratio of 1:2 indeed resulted in a clear aqueous solution (Fig. S1). Converting

the antibiotics into a salt form would also increase their solubility at the deposition site and accordingly the efficacy of the treatment, as the local drug concentration will be increased [10, 49].

The difference between the pK_a values of an acidic and a basic compound gives a general estimate of salt formation if ΔpK_a is above 2 or 3 [50]. The ΔpK_a (Azi&NAC) = 8.61, ΔpK_a (Tobra&NAC) = 8.72 and ΔpK_a (Cipro&NAC) = 4.86 strongly suggests possible salt formation. All these antibiotics and NAC formulations were successfully sprayed and available as DPI formulation. To counterbalance the hygroscopic properties of NAC, L-leucine was added to the formulations as it has previously been shown to shield hygroscopic dry powders from wetting and therefore early disintegration [51]. This effect can be explained by the lipophilic *tert*-butylic tail of L-leucine which strengthens the hydrophobic properties of formulations, prevents water to adsorb at the particle surface and to penetrate in the deeper layers of the particles [51]. All three formulations of antibiotic/NAC displayed similar handling properties with respect to collecting and storing. This implies low moisture content and a low tendency towards aggregation. As illustrated in Fig. 3, all formulations consisted of mostly spherical particles. Cipro/NAC and Azi/NAC formulations exhibited smoother surfaces than the Tobra/NAC formulation, which had a raisin-like appearance.

3.2. Solid state characterization

3.2.1. Solid-state form

The solid-state forms of the three spray-dried DPI formulations were determined with XRPD. This approach gives initial information about the degree of amorphousness (or otherwise) of the samples [52]. The diffractograms of the DPI formulations showed a halo pattern without peaks (Fig. 4a). This halo pattern is a characteristic property of amorphous solids and it is an indication that all the DPI formulations were amorphous after spray drying.

3.2.2. Glass-transition temperature (T_g)

The T_g is another characteristic property of amorphous solids and indicates a change from the glassy state to the supercooled liquid (rubbery) state. The T_g is a second order thermal event and usually determined as a sigmoidal step change in heat flow (or heat capacity) in mDSC thermograms [32, 53]. The T_g s of the neat amorphous starting materials, except for LEU, which could not be amorphized (thermograms not shown), and the DPI formulations (Fig. 4b) were determined and the results are shown in Table 2.

In Fig. 4b, a single sigmoidal step change in the reversing heat-flow thermogram can be observed for the DPI formulations. This is an indication that co-amorphous systems have been obtained [36]. For co-amorphous systems, their T_g values are concentration-dependent and the values are usually between those of the pure starting materials [42, 54]. However, for Cipro/NAC, the experimental T_g (93.3 °C) was similar to that of neat amorphous ciprofloxacin (90.5 °C). This indicates that different amorphous phases (a Cipro-rich and a NAC-rich phase) are present and that Cipro/NAC does not form a co-amorphous system.

For Azi/NAC and Tobra/NAC that formed co-amorphous systems, their T_g s were compared to theoretical values based on the Fox equation (contribution of LEU to the predicted T_g was neglected as its concentration was low). The theoretical values (shown in Table 2) are significantly lower compared to the experimentally determined values. A positive deviation of the experimentally determined values from theoretical values indicates molecular interactions between the drug and the co-former, and from the pK_a differences between the two drugs and NAC (ΔpK_a (Azi&NAC) = 8.61, ΔpK_a (Tobra&NAC) = 8.72), the molecular interaction is very likely to be due to salt formation [50].

3.3. *In vitro* aerodynamic properties

The aerodynamic properties of particles are an important criterion for novel formulations for inhalation. Particles with a diameter bigger than 5 μm are described to mainly deposit in the oropharynx and large conducting airways, whilst particles ranging from 1 to 5 μm predominantly deposit in the small airways and alveoli [55]. Thus, the aerodynamic properties

of the DPI formulations were investigated using the Next Generation Impactor (NGI). The data obtained, such as the mass median aerodynamic diameter (MMAD), the geometric mean (GSD), and the fine particle fraction (FPF) are depicted in Table 3. All formulations showed a high FPF of at least 60% and comparable MMAD values ranging from 2.16 up to 2.63 μm . The multifunctional powder formulations thus exhibited appropriate aerodynamic properties for pulmonary application such as for chronic lung infections in CF. Other factors playing a role for the development of an optimal dry powder formulation (*e.g.*, powder flowability and hygroscopicity) will be investigated in the future.

3.4. Disintegration behavior of the multifunctional DPI formulations in humid air

For powder formulations intended for inhalation, an appropriate disintegration rate of the matrix is crucial. Upon deposition into the lungs, a rapid disintegration of the final product in the available (low) amount of fluid is desired [56]. The combination of the antibiotics with NAC already revealed a positive effect of the salt formation, as drugs with low solubility (azithromycin and ciprofloxacin) became more water-soluble. To investigate their dispersibility, the powder formulations were applied onto a filter membrane at 100% RH; then the membrane was placed on an agarose gel and stored at 37 °C. The fate of the particles on the membrane was visualized using SEM. As a control, spray-dried azithromycin alone without NAC was used. Spray drying of tobramycin and ciprofloxacin alone was not possible without the presence of NAC and could therefore not be used as controls. In comparison to spray-dried free azithromycin without NAC, the powder formulation containing Azi/NAC started to dissolve immediately ($t = 5 \text{ min}$) after placing them on the agarose gel while being mounted on the nitrocellulose membrane (Fig. 5). The spray-dried pure azithromycin did not dissolve up to 30 min (Fig. 5). These results confirm that the presence of NAC in the multifunctional DPI formulation improved solubilization of the antibiotic based on the salification process [49]. In the case of Tobra/NAC and Cipro/NAC, the DPI formulations also showed a fast disintegration

(Fig. S2). Moreover, we have noticed that cleaning the spray drier after the experiments for formulations containing NAC (and in contrast to spray-dried pure azithromycin without NAC) could easily be done only using pure water, further indicating improved solubility of the NAC/antibiotic complex formed.

3.5. Effect of multifunctional DPI formulation on the rheological properties of mucus

Mucus and especially the dense viscous mucus in CF reduces the permeability of drugs and drug carriers. Therefore, a mucolytic agent such as NAC is used to fluidize the mucus and thus enhance permeation [57]. The viscosity of pulmonary horse mucus before and after applying the multifunctional formulations was measured, to assess NAC's influence on the rheological properties of mucus. Mucus was appropriately diluted with pure water to account for the dilution effect introduced by applying the dissolved DPI formulations and was taken as control. When pure NAC solution was added to mucus, a pronounced reduction in the viscosity from 60 Pa s to 18 Pa s (Fig. 6) was observed, due to NAC's ability to cleave disulfide bonds between mucins [27]. Adding the multifunctional powder formulations onto the mucus also led (within 5 min) to a reduction in the viscosity of mucus from 60 to 19 Pa s. These results confirm the efficacy of NAC as mucolytic agent when incorporated in the DPI formulations (Fig. 6).

3.6. Stability of spray-dried multifunctional DPI formulations

The freshly prepared amorphous DPI formulations were stored at 65 °C for six weeks and at 125 °C for twenty minutes to investigate storage stability in the glassy and supercooled liquid form, respectively. The physical stability for Cipro/NAC, determined by XRPD, is shown in Fig. 7A. The diffractograms show complete recrystallization after storage under both storage conditions with clearly identifiable peaks belonging to either ciprofloxacin or LEU or both. SEM images before and after storage also showed differences in surface morphology of the particles, again indicating recrystallization (Fig. S3).

The physical stability of the co-amorphous Azi/NAC and Tobra/NAC formulations is depicted in Fig. 7B, C. The amorphous halo is maintained, both, in the glassy or supercooled liquid form of the formulations. The peaks observed at 6.0 and 19.5° (2 θ), for formulations stored at 125 °C, are characteristic peaks of LEU. The morphology, determined using SEM, of Azi/NAC and Tobra/NAC co-amorphous DPI formulations also did not change as a function of storage. In summary, the co-amorphous DPI formulations are physically stable under stress storage conditions and by extrapolation, will be stable at RT.

3.7. *In vitro* evaluation of the effect of multifunctional DPI formulations against *P. aeruginosa* biofilm

P. aeruginosa biofilm is a community of bacterial cells embedded in a mixture of biopolymers, *e.g.*, polysaccharides, proteins, lipids, and extracellular DNA (eDNA) forming the biofilm matrix [58]. These extracellular polymeric substances have structural, nutritional, and protective functions in the biofilm. The eDNA component of the biofilm matrix plays an important role in biofilm formation, virulence as well as antibiotic resistance [59-61]. We studied the effect of the multifunctional DPI formulations on biofilm formation of the highly virulent clinical isolate *P. aeruginosa* PA14 using the crystal violet (CV) biofilm and eDNA assays [43-45]. The multifunctional DPI formulations containing different concentrations of the antibiotic were screened to determine the appropriate concentration that reveals the impact of the DPI formulations on biofilm, as at high concentration of antibiotic, potential effects can be concealed by the inhibition of bacterial growth. Neat antibiotics and NAC at the same concentration as in multifunctional DPI formulations were used as references. Results revealed that Azi/NAC formulation reduced the biofilm by 25% ($p < 0.0001$ compared to control and azithromycin alone) at a concentration of 0.3 $\mu\text{g/mL}$, while neither azithromycin nor NAC separately showed significant inhibition (Fig. 8). At 10 $\mu\text{g/mL}$, the Azi/NAC formulation displayed 40% inhibition ($p < 0.001$ compared to control) slightly less than azithromycin alone (55%, $p < 0.0001$) (Fig. 8). This might be attributed to a difference in the rate of dissociation to

the free base between azithromycin and the Azi/NAC salt in the formulation at this concentration [62]. No significant difference was observed between ciprofloxacin and the Cipro/NAC formulation. They exhibited about 65% inhibition ($p < 0.0001$ compared to control) at a concentration of 0.1 $\mu\text{g/mL}$, and 88 % suppression at 0.3 $\mu\text{g/mL}$ ($p < 0.0001$ compared to control) (Fig. 8). The equipotent effects of ciprofloxacin and Cipro/NAC formulation can be ascribed to the growth inhibitory effect of ciprofloxacin at concentrations of 0.1 and 0.3 $\mu\text{g/mL}$, which are corresponding to 2- and 6-fold MIC values, respectively (Table S1). At 0.1 $\mu\text{g/mL}$, no inhibition of biofilm was observed for tobramycin, NAC, and the corresponding formulation (Fig. 8). Interestingly, Tobra/NAC formulation showed 70% reduction of biofilm ($p < 0.0001$ compared to control and $p < 0.001$ compared to tobramycin only) at a concentration of 0.3 $\mu\text{g/mL}$ versus 35% ($p < 0.001$) or no inhibition for each ingredient alone, respectively (Fig. 8). At 10 $\mu\text{g/mL}$, both tobramycin and Tobra/NAC formulation exhibited almost full inhibition of the biofilm (95%, $p < 0.0001$ compared to control) (Fig. 8). The latter similar effects could be attributed to the high antibiotic concentration as mentioned above. It is noteworthy that NAC concentrations in this study range from 0.049 $\mu\text{g/mL}$ to 17 $\mu\text{g/mL}$ in Cipro/NAC (0.1 $\mu\text{g/mL}$) and Tobra/NAC (10 $\mu\text{g/mL}$) DPI formulations, respectively. These concentrations are much lower than the reported values for NAC (0.5 – 80 mg) needed to inhibit the biofilm formation in *P. aeruginosa in vitro* [27, 29]. This could explain the lack of activity for NAC alone and the substantial effect in combination with antibiotics against the biofilm. Nevertheless, the significant enhancement of activity for Azi/NAC and Tobra/NAC formulations at 0.3 $\mu\text{g/mL}$ compared to those of the separate components indicates the potential advantages of the combinations. In addition, further optimization of the antibiotic/NAC ratio might be needed to augment the antibiofilm activity.

The inhibitory effects of the antibiotic/NAC formulations on eDNA were in line with those on the whole biofilm. Remarkably, the effect of NAC was more pronounced on the eDNA component alone (38 – 54% inhibition, Fig. S4) compared to a rather slight activity in the whole

biofilm assay at the tested concentrations. Interestingly, we observed a significant increase (18 – 46%, $p < 0.05$ compared to control) in the biofilm and eDNA formation for azithromycin at 0.3 $\mu\text{g/mL}$ and tobramycin at 0.1 $\mu\text{g/mL}$ (Fig. 8 and S4). It has been reported that this phenomenon occurs when antibiotics are present in concentrations below MIC levels [63, 64]. On the other hand, we did not observe such induction for the DPI formulations having the same concentration of antibiotics. Taken together, these results demonstrate that the new antibiotic/NAC combinations in the formulation could have beneficial effects or at least maintain the antibiotic and NAC properties for biofilm inhibition.

3.8. Effect of multifunctional DPI formulations on antibiotic susceptibility

To investigate whether the interaction of NAC with the antibiotics would modulate their antibacterial activity, we tested the antibiotic/NAC formulations, the neat antibiotics, and NAC against *P. aeruginosa* PA14 using the bacterial growth-inhibition assay [46, 47]. Pure NAC did not show any inhibition of *P. aeruginosa* PA14 growth in the concentration range of 0.049 to 0.52 $\mu\text{g/mL}$ corresponding to those in the tested formulations. No significant difference was observed between the growth-inhibitory activities in the presence and absence of DPI formulations for azithromycin and tobramycin (Table S1). Susceptibility to ciprofloxacin in the formulation also did not change (MIC values: 0.05 $\mu\text{g/mL}$ versus 0.06 $\mu\text{g/mL}$ for the neat antibiotic). Altogether, the antibiotic/NAC combination in the DPI formulations did not show significant alteration of the antibacterial activities compared to the neat antibiotics and the MIC values against *P. aeruginosa* PA14 are in accordance with previous findings [65, 66].

4. Conclusions

Respirable multifunctional DPI formulations composed of antibiotics and NAC as matrix were successfully developed. Azi/NAC and Tobra/NAC formulations were found to be co-amorphous salt formulations. Salt formation between the antibiotics and NAC allowed for improving the solubility of the antibiotics. Furthermore, it also enabled the successful formation of DPI formulations using spray drying. Physical stability experiments indicated that the

formulations retained their amorphous form after storage under stress conditions. The shelf-stability of these multifunctional DPI formulations was investigated with respect to their morphology and agglomeration behaviour, revealing stable DPI formulations for azithromycin/NAC and tobramycin/NAC over a range of nine months. The salt formation may also have contributed to a reduced hygroscopicity of the DPI formulations and a positive effect on the redispersibility of the dry powder formulations at 100% RH. In addition to these physico-chemical aspects, the obtained multifunctional DPI formulations of antibiotic/NAC displayed suitable aerodynamic properties with fine particle fractions above 65%. Besides promising deposition behavior, the application of NAC reduced mucus viscosity. Furthermore, the co-formulated antibiotics allow a direct treatment of residing bacteria as the antibiotic/NAC DPI formulations improved or at least maintained both, antibiotic susceptibility and NAC inhibitory properties on *P. aeruginosa* PA14 biofilms. The combination of antibiotics and NAC, exploiting co-amorphous salt formation has allowed us to realize a novel multifunctional DPI formulation and this knowledge may provide the basis to formulate a variety of drug combinations for inhalation as dry powders.

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Table Headings

Table 1: Amount of NAC and antibiotic in the spray drying solution with the needed percentage of L-leucine.

Table 2: Glass transition temperatures (T_g s) of the neat amorphous starting materials and the multifunctional DPI formulations.

Table 3: Results of the NGI evaluation of the DPI formulations.

Figure Captions

Fig. 1: SEM micrograph of spray-dried pure NAC.

Fig. 2: Ionization site of antibiotics and NAC for the multifunctional DPI formulations.

Fig. 3: SEM micrographs of multifunctional DPI formulations. Azi/NAC (A), Cipro/NAC (B), Tobra/NAC (C).

Fig. 4: a) Diffractograms and b) thermograms (mDSC) of freshly prepared DPI formulations. Arrows in the thermograms show the sigmoidal step change in the reversing heat flow signal, indicating the glass transition temperature.

Fig. 5: SEM micrographs of the DPI formulation deposited on top of a nitrocellulose membrane. (A) Spray-dried azithromycin without NAC and (B) DPI formulation Azi/NAC at 5 min after being placed on top of agarose pads up to 30 min.

Fig. 6: Effect of multifunctional DPI formulations containing NAC on the viscosity of mucus.

Fig. 7: Diffractograms of stored antibiotic/NAC samples. (A) Cipro/NAC samples showing recrystallization of ciprofloxacin (red arrows) and LEU (green arrow). A combined diffractogram of crystalline NAC, LEU and ciprofloxacin is shown for comparison. (B) Diffractogram of the co-amorphous Azi/NAC and (C) Tobra/NAC samples after storage at 65 °C for 6 weeks and 125 °C for 20 min. The diffractogram of the crystalline LEU is shown for comparison in both diagrams.

Fig. 8: Effects of the multifunctional DPI formulations at different concentrations on biofilm formation in *P. aeruginosa* PA14. Neat antibiotics and NAC at the same concentrations as in the multifunctional DPI formulations were used as references. ns, not significant; * $p < 0.05$; *** $p < 0.001$; and **** $p < 0.0001$ indicate a significant difference (t -test) compared to control.

Table 1: Amount of NAC and antibiotic in the spray drying solution with the needed percentage of L-leucine.

Formulation	NAC [w%]	Antibiotic [w%]	L-Leucine [w%]
Azi/NAC	28.83	66.17	5
Cipro/NAC	29.7	60.3	10
Tobra/NAC	57.22	32.78	10

Table 2: The T_g s of the neat amorphous starting materials and the multifunctional DPI formulations.

<i>Sample Name</i>	<i>Experimental T_g</i>	<i>Predicted T_g based on Fox equation</i>
Azi/NAC	99.0 ± 0.9	66
Azithromycin (QC)	108.3 ± 0.5	-
Tobra/NAC	107.9 ± 0.7	38
Tobramycin (QC)	135.5 ± 0.1	-
Cipro/NAC	93.3 ± 0.4	50
Ciprofloxacin (BM)	90.5 ± 0.1	-
N-acetyl-cysteine (QC)	6.9 ± 1.9	-

- Amorphous N-acetyl-cysteine, tobramycin and azithromycin were prepared via quench cooling (QC)
 - Ciprofloxacin was made amorphous prepared by ball milling (BM)

Table 3: Results of the NGI evaluation of the DPI formulations.

	Cipro/NAC	Tobra/NAC	Azi/NAC
MMAD [μm]	2.40 (± 0.02)	2.16 (± 0.33)	2.63 (± 0.03)
GSD	1.88 (± 0.008)	1.53 (± 0.06)	1.64 (± 0.13)
FPF [%]	61.60 (± 5.23)	97.52 (± 15.36)	67.40 (± 9.41)

SD is provided in brackets

Fig. 1: SEM micrograph of spray-dried pure NAC.

Fig. 2: Ionization site of antibiotics and NAC for the multifunctional DPI formulations.

Fig. 3: SEM micrographs of multifunctional DPI formulations. Azi/NAC (A), Cipro/NAC (B), Tobra/NAC (C).

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Spray-dried azithromycin without NAC

A

Spray-dried azithromycin with NAC

B

Fig. 5: SEM micrographs of the DPI formulation deposited on top of a nitrocellulose membrane. (A) Spray-dried azithromycin without NAC (B) DPI formulation Azi/NAC at 5 min after being placed on top of agarose pads up to 30 min.

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Fig. 8: Effects of the multifunctional DPI formulations at different concentrations on biofilm formation in *P. aeruginosa* PA14. Neat antibiotics and NAC at the same concentrations as in the multifunctional DPI formulations were used as references. ns, not significant; * $p < 0.05$; * $p < 0.001$; and **** $p < 0.0001$ indicate a significant difference (*t*-test) compared to control.**

Supporting Information

Spray-drying of inhalable, multifunctional formulations for the treatment of biofilms formed in cystic fibrosis

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Fig. S1: The dry substances of Tobra/NAC, Cipro/NAC & Azi/NAC respectively, show differences in dissolving speed upon contact with water. Cipro/NAC takes longer to completely dissolve than the other two formulations. Finally, all formulations were clear solutions after 1h. Picture was taken 1 min after contact.

Fig. S2: SEM micrographs of multifunctional DPI formulations deposited on top of a nitrocellulose membrane. (A) formulation on top of the membrane without agarose pad right after deposition, (B) DPI formulations 5 min after being placed on top of agarose pads, (C) DPI formulations 30 min after being placed on top of agarose pads.

A

B

C

Fig. S3: SEM micrographs of multifunctional DPI formulations (A) Cipro/NAC, (B) Azi/NAC, (C) Tobra/NAC after 3 months.

Table S1. Antibacterial activities of the neat antibiotics and the antibiotic/NAC DPI formulations.

Antibiotic	Concentration ($\mu\text{g/mL}$)	<i>P. aeruginosa</i> PA14 % inhibition of growth	
		neat	DPI Formulations
Azithromycin	0.3	25 ± 5	36 ± 1
Ciprofloxacin	0.003–0.1	0.06^a	0.05^a
Tobramycin	0.1	n.i. ^b	n.i.
Tobramycin	0.3	10 ± 2	10 ± 4

^aMIC values ($\mu\text{g/mL}$) were calculated; ^bn.i. no inhibition.

Fig. S4: Effects of the DPI formulations at different concentrations on eDNA formation in *P. aeruginosa* PA14. Neat antibiotics and NAC at the same concentrations as in the multifunctional DPI formulations were used as references. ns, not significant; ** $p < 0.01$; *** $p < 0.001$; and **** $p < 0.0001$ indicate a significant difference (*t*-test) compared to control.

Fig. S5: Thermograms showing the T_g s of neat amorphous n-acetyl cysteine, tobramycin, azithromycin and ciprofloxacin.