In vitro inhalation cytotoxicity testing of therapeutic nanosystems for pulmonary infection

Detlef Ritter,⁎, Jan Knebel, Monika Niehof, Iraida Loinaz, Marco Marradi, Raquel Gracia, Yvonne te Welscher, Cornelus F. van Nostrum, Chiara Falciani, Alessandro Pini, Magnus Strandh, Tanja Hansen

Preclinical Pharmacology and In-vitro Toxicology, Fraunhofer ITEM, Hannover, Germany
CIDETEC Nanomedicine, San Sebastián, Spain
Utrecht University, Utrecht, the Netherlands
SetLance, Siena, Italy
Adenium Biotech ApS, Copenhagen, Denmark
Department of Medical Biotechnology, University of Siena, Italy

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ABSTRACT

Due to the increasing need of new treatment options against bacterial lung infections, novel antimicrobial peptides (AMPs) are under development. Local bioavailability and less systemic exposure lead to the inhalation route of administration. Combining AMPs with nanocarriers (NCs) into nanosystems (NSs) might be a technique for improved results.

An air-liquid interface (ALI) in vitro inhalation model was set up including a human alveolar lung cell line (A549) and an optimized exposure system (P.R.I.T.® ExpoCube®) to predict acute local lung toxicity. The approach including aerosol controls (cupper-II-sulfate and lactose) delivered lowest observable adverse effect levels (LOAELs).

Different combinations of AMPs (AA139, M33) and NCs (polymeric nanoparticles (PNPs), micelles and liposomes) were tested under ALI and submerged in vitro conditions.

Depending on the nature of AMP and NCs, packing of AMPs into NSs reduced the AMP-related toxicity. Large differences were found between the LOAELs determined by submerged or ALI testing with the ALI approach indicating higher sensitivity of the ALI model. Since aerosol droplet exposure is in vivo relevant, it is assumed that ALI based results represents the more significant source than submerged testing for in vivo prediction of local acute lung toxicity.

In accordance with the current state-of-the-art view, this study shows that ALI in vitro inhalation models are promising tools to further develop in vitro methods in the field of inhalation toxicology.

1. Introduction

Infections of the lower respiratory tract (LRTI) are usually triggered by infection or penetration of pathogenic microorganisms (Zhang et al., 2018). LRTIs, which include acute bronchitis, bronchiolitis, influenza and pneumonia, are a significant cause of disease and death in patients worldwide (Feldman and Richards, 2018). Antibiotic therapy is a key factor in the treatment plans of these diseases. The dramatic increase in antimicrobial resistance among respiratory pathogens is a problem worldwide. The World Health Organization has identified antimicrobial resistance as one of the three major threats to human health (Vishwanath et al., 2013). To treat infections, antibiotics are commonly administered systemically, such as by oral or intravenous route. In the case of LRTI, administration by inhalation may offer some beneficial characteristics, such as deposition of the unmodified antimicrobial substance directly at the location of infection and a less intense systemic exposure to avoid severe side effects in other organs (Hatipoglu et al., 2018). In the context of the EU project PneumoNP (7th Framework Program, grant #604434), new nanosystems (NSs) were developed, including novel combinations of antimicrobial peptides (AMPs) within a nano-sized packaging made of different materials (nanocarriers, NCs) such as polymeric nanoparticles (PNPs), liposomes or micelles. By loading into nanosystems, the stability of...
the AMPs during and after administration was expected to be improved while their toxicity was expected to be reduced. During development, toxicity testing of inhalable pharmacologically active substances is essential at an early stage to exclude candidates with disadvantageous characteristics. Hence, test systems are needed which offer the possibility of a relevant procedure under routine conditions and lead to significant results. In vitro systems with cells of human origin have been applied in the recent years for many approaches, including lung toxicity testing. Due to the physiological principles of inhalation these approaches are facing additional challenges to realize relevant testing scenarios by cell exposures to the atmosphere and the inhalable test material in its relevant formulation as gas, vapor or aerosol. Air-liquid-interface (ALI) cell culture systems have been applied to explore the inhalable effects of volatiles (Al Zallouha et al., 2017), aerosols (Svensson et al., 2016; Jing et al., 2015), complex aerosols such as e-cigarette smoke (also indicating in vivo relevance of the results) (Moses et al., 2017), or aerosols that might be produced from commercial products such as in the area of cosmetic products (Ritter et al., 2018). The cell culture-based systems are an important part of new animal-free testing strategies in the sense of the 3R (European Union, 2010; Russell and Burch, 1959). Especially the possibility to test the inhalable development candidate in its final formulation where only limited amount of test material is available is of great benefit. This was also true for the present case. Two different antimicrobial peptides (AA139: Lee et al., 2007; Edwards et al., 2016; Hoegenhaug et al., 2011 and M33: Brunetti et al., 2016a; 2016b; van der Weide et al., 2017) in combination with three different NC types (micelles, liposomes, PNPs) were under development. The purpose of the current study was to develop an in vitro testing procedure for these compounds that meets the following demands:

- Application of the final aerosol formulation as delivered from a specific aerosol generator as developed within the PneumoNP project.
- Use of human lung cells in a relevant exposure situation.
- Definition of a strategy for a first, basic evaluation model to classify the results.

To assess the significance of results from the testing procedure, the demonstration of predictivity of the results to the in vivo situation is necessary. This may include two different aspects: on the one hand, the dose-metrics are an important tool to enhance their informational value. By referring the dose-metrics to a culture surface-based dimension such as mass/area [μg/cm²], they are getting comparable to data from in vivo inhalation testing (Schmid and Cassee, 2017; Kim et al., 2014) as a first step in the direction of quantitative in vitro - in vivo correlation (QIVIVE; Mijoung et al., 2015; Tsioun et al., 2016; Naritomi et al., 2015). In inhalation toxicology, such models usually have to be based on the correlation of reference data from e.g. the European Chemicals Agency (ECHA, 2018) or ChemIDplus (ChemID plus, 2018) databanks. However, due to a lack of comparable in vivo inhalation data for nanoparticle encapsulated antimicrobial substances, a different strategy had to be followed, namely the inclusion of positive and negative control compounds in the test setup. Copper sulfate and lactose were identified for this purpose. Additionally, standard controls such as exposure controls, non-exposure controls and vehicle controls were included. This strategy was recently applied in our study on inhalation toxicity testing of aerosols released from a consumer product (Ritter et al., 2018).

Using this approach, selected nanosystems from the project were tested to evaluate applicability of the in vitro testing procedure and potentially different toxicological potencies of the drug candidates. Moreover, as an intermediate technical step to the final ALI in vitro testing setup, a number of the test items were included in a submerged in vitro model, which also included human lung cells but in a standard culture situation with an exposure under submerged culture conditions to solutions of the test items in culture media. The two different in vitro approaches were compared to gain more insight into testing opportunities and to assess the possibly quantitatively or qualitatively different results.

2. Material and methods

2.1. Experimental setup and aerosol generation

The generation of aerosols was based on a setup as developed and delivered through the PneumoNP project by Ingeniatrics (Cossio et al., 2018). It included a nozzle (FB240) driven by compressed air (4.5 bar) and a continuous liquid flow from aqueous solutions of the test substances in buffer. In previous studies, the mean droplet size distributions (MDSDs) were between 2.1 and 2.9μm with 64 to 75% between

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**Fig. 1.** a) Experimental setup for aerosol generation and air-liquid interface (ALI) cell-based in vitro exposures; (1) reservoir for test substances and positive control substances (2) reservoir for vehicle control (3) peristaltic pump, (4) compressed air (5) nebulizer with glass chamber (Ingeniatrics, Spain), (6) excess aerosol, (7) scattering light photometer, (8) P.R.I.T.® ExpoCube®, (9) impingers for washing out the droplets from the aerosol before entering the ControlUnit (10) P.R.I.T.® ControlUnit (11) waste; b) Layout of the 12-well culture plate during ALI exposure of A549 human lung cells.
0.9 and 5.25 μm as determined by laser diffraction and a completely
decayed 2-deoxy-2-(18F)fluoro-o-glucose tracer (Cossio et al., 2018).
The cell exposure was carried out using an optimized exposure device
for ALI cultures in standard 12-well culture insert plates (P.R.I.T.* Expo-
Cube®, Ritter and Knebel, 2014). It was positioned in-line to the
aerosol generation (Fig. 1) and enabled a concurrent test aerosol ex-
posure, vehicle control exposure and a non-exposure control. The
aerosol generation was monitored by scattering-light photometers
positioned at the inlets of the ExpoCube®.

2.2. Test compounds

2.2.1. Selection of controls

According to their known toxicological characteristics as “non-
toxic” respectively “mild toxic and irritative” substances and the ap-
\nlication under the test conditions (water solvability, usability in
\nnebulizer, analytical accessibility for deposition measurements)
lactose (Sigma-Aldrich, Germany, L3750) and copper-II-sulfate (Roth,
\nGermany, CP86.2) were selected as negative and positive controls,
respectively.

For the submerged exposure, a "toxic polymer" (poly(2-dimethyl-
aminomethyl methacrylate), CIDETEC, San Sebastian) with a well-known
toxic behavior was chosen to validate the functionality of the assay in
testing the specific group of candidates.

2.2.2. Test items

AMPs were loaded into different NCs to form NSs and tested by aero-
sol / ALI testing and using a conventional submerged in vitro ap-
\nproach. AA139 was combined with PNP s and micelles (AA139-PNP,
AA139-Mic) and M33 was combined with PNP s and loaded into a lipid
nanostructure (M33-PNP, M33-Lip). Table 1 illustrates the composition of
the different test items. The lipid nanocompound in the “AA139-Mic”
NS consisted of 1,2-distearoyl-sn-glycero-3-phosphoethanolamine con-
jugated polyethylene glycol (DSPE-PEG2000). The lipid nanocompound
in the “M33-Lip” NS consisted of a 10:1:0.6 mixture of egg phosphati-
dylcholine (EPC), egg L-α-phosphatidylglycerol (EPG) and DSPE-
PEG2000, respectively.

2.3. Cell culture and viability measurements

A human lung epithelial cell line A549 (ATCC® CCL-185®) was
obtained from ATCC (LGC Standards GmbH, Germany). Cells were rou-
\ntinely cultured in 75 cm² flasks in Dulbecco’s modified Eagle’s me-
dium (DMEM) supplemented with 10% fetal calf serum (Biochrom
\nGmbH, Germany) and 0.01% Gentamicin at 37°C in a humidified at-
\nmosphere containing 5% CO₂. For cytotoxicity testing, subconflu-
ent cultures were trypsinised and cell viability was determined using an
electronic cell counter (CASY, Schärfe Systems, Reutlingen, Germany).

2.4. Cell exposure

For submerged exposures, A549 cells were seeded into 96-multiwell
plates at a density of 8 × 10⁴ per well. Prior to treatment with test
items, cells were allowed to proliferate to reach 70–80% conflu-
ence. Before exposure, culture medium was changed to DMEM without fetal
calf serum. Test items were added and a tetrazoilum salt cleavage based
cell viability assay (WST-1) was performed after 4 h and 24 h.

For air-liquid interface culture an aliquot of 4x10⁴ A549 cells was
seeded on microporous membranes (Inserts, BD Falcon; 0.4 μm pore
size; growth area ~1 cm²). Cells were further cultivated on the mem-
branes for approximately 72 h until they reached a confluent monolayer
as inspected by light microscopy. At 16-18 h before exposure, the cell
medium was replaced with serum-free DMEM. Just prior to the ex-
posure with the test substances, residual liquid from the apical side of
each cell monolayer was gently removed. During the treatment, cells
were nutrited by culture media from beneath the membrane while
being exposed to the air or aerosols from the top. Cell exposure was
carried out using the P.R.I.T.® ExpoCube® using exposure flows of 3 ml/
min and exposure times between 15 and 60 min. The setup is schema-
tically described in Fig. 1.

2.5. Estimation of cellular viability

The test was performed using a WST-1 assay kit from Roche
(Mannheim, Germany). For the measurement of WST-1 reduction in 96
well plates, cell culture plates were incubated with the test items in
100 μl medium and were then used for measurement of cyto-
toxicity. After incubation, the incubation medium was removed and cells
were incubated for 30 min at 37°C in a mixture a 100 μl culture medium and
10 μl of freshly prepared WST-1 solution per well. The absorbance of
the formazan solution was then determined at 450 nm with a reference
wavelength of 630 nm using a SpectraMax 340PC spectrophotometer
(Molecular Devices, Ismaning, Germany).

Following to exposure of A549 cells on membranes to aerosols from
test items or positive or negative control substances, measurement of
tetrazoilum salt conversion was carried out. Therefore, cells were in-
\ntubated for 60 min at 37°C with 500 μl of medium from the apical side
\ncontaining 10% WST-1 solution per membrane. Aliquots of 100 μl were
\ntransferred into 96-well microplates for measuring their absorbance at
\n450 nm with a reference wavelength of 630 nm.

2.6. Determination of dosages

To characterize the application of dosages during aerosol exposure
in the setup quantitatively, copper-II-sulfate was used and aerosolized
from solution (5% in water). Empty culture inserts were exposed to the
aerosol under the same conditions as used for cell exposure. After ex-
posure, the culture membranes were rinsed with 200 μl water and these
samples were analyzed using a copper-II-spectrophotometric test kit

Table 1
Test items and compositions for aerosol / ALI and submerged in vitro tests. Solvents were 0.9% sodium chloride or HEPES-buffered saline (HBS). Dilutions for submerged tests were made in culture media.

| Test item | AMP composition for aerosol / ALI testing | NS composition for submerged testing |
|-----------|----------------------------------------|-------------------------------------|
|           | conc. [mg/ml] | conc. [mg/ml] | conc. [mg/ml] | NS | Composition |
| AA139-free | 12.70 | – | – | 46.60 | Dilutions from pure AA139 |
| AA139-Mic | 12.60 | 34.00 | – | 44.00 | Dilutions from NS (10.4 mg AA139 / 32.6 mg micelles) |
| AA139-PNP | 4.00 | 40.00 | – | 45.60 | Dilutions from NS (0.1 mg AA139 / 1 mg PNP) |
| M33-free | – | – | – | 60.00 | Dilutions from NS (3.2 mg M33 / 83.26 mg lipids) |
| M33-PNP | 5.60 | 40.00 | – | 60.00 | Dilutions from NS (3.2 mg M33 / 83.26 mg lipids) |
| M33-Lip | 1.70 | 58.30 | – | – | – |

1) Not tested.
(Merck, Darmstadt, 114,553) according to the instructions of the manufacturer. Dosages applied during cell exposures to copper-II-sulfate and any other test item were calculated from these representative deposition experiments using copper-II-sulfate as a tracer.

2.7. Statistical analysis and calculation of LOAELs

Percentage of control values were calculated from tetrazolium-salt WST-1 assay data after transfer to Excel (Microsoft Office 13). Dose response fitting was carried out using Origin 2018 (OriginLab Corporation) according to a best-fit strategy with upper and lower confidence intervals (95%). Variation of controls was estimated by calculation of standard deviations of the controls from repeated exposure experiments. As lowest observed adverse effect level (LOAEL) the lowest dosage was considered, where the statistical variation of the dose-response curve (upper confidence level, UCL) was significantly different from the variation of the controls (100% - standard deviation).

3. Results

3.1. Development of ALI aerosol generation and cell exposure setup

3.1.1. Quantitative determination of droplet deposition

For a quantitative determination of the amount of test material deposited during cell exposure, empty culture membranes were exposed under relevant cellular exposure conditions to aerosol from a 5% copper-II-sulfate solution. 5% test item concentration was the technically highest possible concentration for the antibiotic nanosystems. Using exposure times between 5 and 30 min and a liquid flow of 450 μl/min with the generation unit, a deposition rate of 0.4 μg/cm² test material per minute on the exposed surface was evaluated (Fig. 2, left) by chemical analysis of the deposited copper. Moreover, there was no statistical difference between the four technical replicates, which were exposed to the aerosol in parallel in each single experiment (Fig. 2, right).

3.1.2. Exposure controls and non-exposure controls

Exposure controls, vehicle controls and non-exposure controls were processed in a concurrent way during the exposure experiments in the same multi-well plate. Fig. 3 shows a representative plot from 23 exposure experiments. The basic cell viability as analyzed by tetrazolium salt cleavage was very stable in the non-exposure controls over the experimental period. The exposure towards the vehicle control (aerosol solution as negative or positive controls, respectively. Different dosages were calculated from mass-based dosages according to the results from droplet deposition experiments as described above. Fig. 4 shows the results of the experiments as percentage of control values (vehicle control exposure aerosols contained 0.9% NaCl or PBS). A dose-response relationship was found for the positive control copper-II-sulfate with an EC₅₀ value of 11.4 μg/cm² with a lowest observed adverse effect level (LOAEL) of 4.6 μg/cm². In contrast, exposure to lactose aerosols did not induce significant toxicity up to highest dosages of 90 μg/cm².

3.1.3. Exposure to positive and negative controls

A549 cells were exposed to aerosols of lactose or copper-II-sulfate solutions as negative or positive controls, respectively. Different dosages were achieved by combining different compound concentrations (lactose 5 to 20%, copper-II-sulfate 5%) and exposure times (10 to 60 min). Deposited dosages were calculated from exposure times, test item concentrations and the results from droplet deposition experiments as described above. Fig. 4 shows the results of the experiments as percentage of control values (vehicle control exposure aerosols contained 0.9% NaCl or PBS). A dose-response relationship was found for the positive control copper-II-sulfate with an EC₅₀ value of 11.4 μg/cm² with a lowest observed adverse effect level (LOAEL) of 4.6 μg/cm². In contrast, exposure to lactose aerosols did not induce significant toxicity up to highest dosages of 90 μg/cm².

3.1.4. Establishment of experimental design

Based on the copper-II-dose response curve, an experimental design was defined. This included the definition of three exposure groups “high”, “medium” and “low” for the testing of the aerosols generated from test items. To reference the experimental design quantitatively to the positive control copper-II-sulfate as a substance with a mild inhalation toxicity in human, the dose-ranges for the test items were defined based on EC₇₅- and EC₁₅-values (6.5 and 17 μg/cm², respectively) from the respective dose response relationship as depicted in Fig. 5. The dosage in the highest dosage group (dosage ≥17 μg/cm²) represents the highest dosage for each individual test item according to the respective technical limitations (e.g. solvability or stability of test items).

3.2. Exposure to test items

3.2.1. Aerosol (ALI) in vitro exposure test setup

A549 cells were exposed to aerosols containing the test items in the three dosage groups “low”, “medium” and “high” in three to five independent experiments for each dosage group. Fig. 6 shows the results in two ways. The bar graphs document the results as a function of the grouping of experiments. Dose response plots in turn show the results after calculation of mass-based dosages according to the results from deposition experiments as described above.

3.2.1.1. AA139-free. Free AA139 was aerosolized and tested without coupling to a nanocarrier. A significant reduction of cell viability was found in the medium and high dose exposure groups and a slight decrease was seen in the low dosage exposure group with a LOAEL of 1.4 μg/cm² (Fig. 6a).

**Fig. 2.** Determination of deposited dose and relative deposition efficiency in the exposure device. a) Results of measurements of the delivered dose on microporous membranes in the cellular exposure situation with copper-II-sulfate aerosols depending on the exposure time. Dots represent results from independent experiments using a liquid flow of 450 μl/min through the nebulizer and a 5% copper-sulfate solution at 5, 10, 20 or 30 min of exposure. b) Mean values of the relative deposition efficiencies on the single culture positions / technical replicates in the exposure device referenced to the mean of the deposition from all deposition experiments with standard deviations.
3.2.1.2. AA139-Mic. The results from cellular toxicity evaluation of aerosolized AA139 micellar nanosystem show that only very slight effects could be detected at the medium and high level exposure groups. The calculation of the LOAEL resulted in a value of 16.6 μg/cm² (Fig. 6b).

3.2.1.3. AA139-PNP. Testing of AA139 after packing into nanosystems with coupling to PNPs resulted in a very slight toxicity only in the highest dosage group (LOAEL ≥ 21.1 μg/cm², Fig. 6c).

3.2.1.4. M33-PNP. M33 was tested as a nanosystem coupled to PNPs in these experiments. The results document a significant cellular toxicity in the medium and high exposure groups in comparison to controls and lactose, corresponding to NS exposure dosages ≥ 9.4 μg/cm² (Fig. 6d). The LOAEL was calculated as 9.0 μg/cm².

3.2.1.5. M33-Lip. M33 was also tested as a nanosystem coupled to a liposomal carrier. Only a very slight toxicity could be detected in the medium and the high exposure groups, corresponding to dosages of 12 μg/cm² and higher, and hence, induced a clearly lower toxicity than the positive control cupper-II-sulfate (Fig. 6e).
Fig. 6. Results from exposures of human lung A549 cells towards aerosols generated from the test items. Left: Results from single experiments based on exposure groups low, medium or high, middle: Dose response relationships based on the calculation of the delivered dose and variability of controls for determination of LOAELs; right: structures of the test items. PNP structures have only illustrative character (c, d).
liquid-interface cultures with human lung cells. Since the first studies microbial peptides. with a group of specific test items including nano-systems with anti- sent aerosol generation device and conduction of a first series of tests mental setup for in vitro inhalation testing under application of a pre- 

4. Discussion

The overall aim of this study was the establishment of an experimen- tial setup for in vitro inhalation testing under application of a pre- sent aerosol generation device and conduction of a first series of tests with a group of specific test items including nano-systems with anti- microbial peptides. Given the aim of an in vivo relevant in vitro inhalation test scenario for droplet aerosols, state-of-the-art methods include the application of air-liquid-interface cultures with human lung cells. Since the first studies exploring the biological effects of inhalable substances back in the 1960s (Pace et al., 1969) and the establishment of the cell culture and cell exposure directly at the air-liquid interface (Voisin et al., 1975, 1976, 1977), numerous in vitro exposure models have been proposed. With respect to the application of aerosols recently the study of inhalable products from e-cigarettes has come into focus (Bathrinarayanan et al., 2018; Iskandar et al., 2017), but the principle has also been used for a variety of other groups of inhalable materials such as influenza virus exposure (Creager et al., 2017). Also, regarding the testing of pharmacologically active substances experimental approaches have been reported such as the testing of nasal sprays (Knebel et al., 2001) or other aerosols (Hein et al., 2010; Lenz et al., 2014). However, these approaches represent technical solutions that usually can only be applied for the individual study. Accordingly, the setup for testing nasal sprays included the use of typical vessels for this purpose, the PADOCC system (Hein et al., 2010) a commercial dry-powder in- haler, and the ALICE-Cloud system (Lenz et al., 2014) a commercial nebulizer system. The application of each of these generation systems is essential for the functionality of the respective experimental setup and cannot be changed to something else. To establish a solution for the pre- sent study, we used the P.R.I.T.* ExpoCube® exposure system (Ritter and Knebel, 2014) which by principle can be adapted to any source of inhal- able material generation, provided the delivery of a 200 ml/min sample flow can be realized. This system was connected to a recently developed aerosol generation system (Cossío et al., 2018). The subsequent inv- estigations focused on (1) the technical (droplet deposition) and biolo- gical (cellular exposure effects from process controls and positive and negative controls) characterization of the inhalation model, (2) the setup of a testing design and its first application to a set of antimicrobial peptides combined with three different nano-compounds, and (3) a parallel testing approach using conventional submerged in vitro human lung cells for a further classification of ALI results.

4.1. Development of ALI exposure setup

An in vitro inhalation model including ALI cultures of a human A549 lung cell line was set up. It included the concurrent exposure of a test aerosol exposure group, a vehicle control exposure group and a non-exposure group in one 12-well tissue culture plate. The P.R.I.T.* ExpoCube® exposure device was combined with a custom-made nebu- lizer system (Ingeniatic; Cossío et al., 2018) and characterized with respect to droplet deposition (Fig. 2), by use of aerosolized CuSO4 so- lutions. The use of this compound was beneficial in two ways. On the one hand, Cu-(II) salts can be analyzed quantitatively by a simple spectrophotometric assay based on the colored cuprizon complex (Rumori and Cerdà, 2003) for dosimetry considerations. On the other
hand, copper-II-sulfate is a mild irritating inhalative toxicant that is known from human epidemiological data based on the “Bordeaux-solution”, which is used during vineyard spraying as a fungicide. It induces severe eye irritation, irritation of respiratory tract, severe irritation of mucous membranes, congestion, lung inflammation, copper deposits, degenerative changes in the lung and sensibilization (Mathew et al., 2015; Thompson et al., 2012; Pimentel and Marques, 1969). Cu-II-sulfate, hence, can serve as a positive substance for in vitro inhalation toxicity testing.

To evaluate the exact dose during each exposure run, the droplet deposition rate as a characteristic of the technical process was evaluated. During a former study (Ritter et al., 2018) this strategy had successfully been applied using dry particle aerosols. The results of that study had shown, that particles had been deposited reproducibly and efficiently in a setup using the ExpoCube®, leading to a particle deposition rate that was independent on the type of material tested (sodium dodecyl sulfate, sodium chloride, lactose, copper sulfate) but dependent on particle size. During this study here, droplet aerosols instead of dry particle aerosols were used. Hence, deposition rates could not be taken from the former results. Instead, exposures to copper-II-sulfate droplet aerosols were carried out using exactly the same experimental conditions as during exposure to NSs later on, with the exception of using a 5% copper-II-sulfate solution instead of NS test items, which were also used in concentrations of up to 5%. As a result of this, aerosol conditions including droplet particle sizes and following droplet deposition rates on the cells were completely identical. Based on this strategy, the use of copper sulfate aerosols under completely identical conditions to the NSs droplet exposure, enabled the realization of the two validation steps, as outline above, at the same time. The evaluation of the droplet deposition rate during aerosol exposure for dosimetry considerations and the use as a slightly toxic positive control during droplet aerosol exposure of cells.

It turned out that 0.4 μg/cm² copper-II-sulfate were deposited on the cellular surface per minute using the system during aerosolization of 5% copper-II-sulfate solutions. The dosage could be defined by adjustment of the concentration of the test item in the solution and the exposure time. By application of copper-II-sulfate as a positive control the responsiveness of the in vitro inhalation model towards human inhalable toxicants could be shown. The EC50 value for copper-II-sulfate exposure of A549 cells was detected at 11.34 μg/cm² (Fig. 4). In an earlier study, where copper-II-sulfate was also applied from an airborne state, but as a dry particle aerosol and not a droplet aerosol, an EC50 value of 23.66 μg/cm² was found (Ritter et al., 2018). Both values indicate a strong cell toxic behavior of this salt. Furthermore, the comparison of these data indicate, that the physicochemical state of the compound during cell exposure might have an effect on the cellular toxicity. In comparison to dry particle exposure, a droplet exposure might induce a stronger effect due to immediate biological availability. This might be an indication that not only the ALI setup is a driver for relevance of results but also the comparability of the physicochemical conditions of the aerosol during exposure.

Moreover, exposures towards aerosols from lactose, concurrent exposures to aerosols from the respective vehicle and process controls such as non-exposure controls, indicated, that there were no significant adverse effects on the cells induced by the exposure scenario.

### 4.2. Exposure design and evaluation of effects

The nature of the test items, namely nanosystems (NSs) composed from antimicrobial peptides (AMPs) and varying nanocarriers (NCs) resulted in two different possibilities for referencing the dosage. The complete nanosystem as the whole test item, defining potentially technical conditions by characteristics such as stability, solubility, physicochemical characteristics for aerosolization, and toxicity (as a resulting toxicity from the NC and the AMP). The included AMP, however, is the relevant component for the intended pharmacological effect and patient treatment, and, hence, its dosage is fundamental for medication. The molecular ratio of NCs and AMPs was very different for the different types of NS. As such, the molecular ratio from NC to AMP was 229:1 for the M33-Lip NS. In contrast, it was 2.5:1 (NC:AMP) for the AA139-Mic NS. To compensate for these differences during the experimental setting, an exposure design was set up based on the dose-response curve from copper-II-sulfate as a positive control for toxicity during ALI testing. It enabled the application of the highest technical possible dosages from the test items based on NS concentration which was in a concentration range clearly above the EC50 value of CuSO4. During submerging tested, the highest dosage was set by a concentration of 500 μg/ml. On the one hand, this is a common highest dosage during these kinds of tests and was selected according to an ICH-Guideline (ICH, 2012). On the other hand, the application of a “toxic polymer” (a potential NC) demonstrated clear toxicity within this dosage range, thus, confirming it as an appropriate range to detect a potential toxic behavior of a test item.

Due to the mainly low effects, which were found within these concentration ranges in both test systems, lowest observed adverse effect levels (LOAELs) were calculated from the dose response data to enable a comparable evaluation of toxic effects from the test item in both test systems and based both on NS dosage and AP dosage.

### 4.3. Comparison of effects during ALI testing

A set of NSs was defined to characterize potential differences in toxicity and respective effects of packaging of AMPs into nanosystems. It included the pure antimicrobial peptide AA139 (AA139-free), the AMP AA139 packed into micelles (AA139-Mic) or packed into polymeric nanoparticles (AA139-PNPs), the antimicrobial peptide M33 (van der Weide et al., 2017) packed into polymeric nanoparticles (M33-PNPs) and packed into liposomes (M33-Lip). Fig. 6 and Table 2 summarize the results from ALI testing. The results clearly demonstrate that packing of AA139 into a NS, composed of micelles or PNPs reduced the toxicity of the AMP. Packing resulted in a toxicity reduction factor of 4.3 (AMP and NS dosage) for the comparison of the LOAEL of free AA139 and AA139-Mic in the ALI test (Table 2, LOAEL(AA139-free, NS) = 3.9 μg/cm², LOAEL(AA139-Mic, NS) = 17 μg/cm², LOAEL(AA139-free, AMP) = 1.4 μg/cm², LOAEL(AA139-Mic, AMP) = 6.2 μg/cm²). LOAEL reduction factors of at least 5 and 1.5 were found based on NS and AMP dosage, respectively, in comparison of AA139-PNP and AA139-free (based on values from Table 2). By comparison to the AA139-PNP NS, the M33-PNP NS showed a clearly higher toxicity both on the basis of NS dosage and AMP dosage (Fig. 6, Table 2). Together with the finding, that the toxicity of the free M33 was higher than of the free AA139 (lower LOEAL of M33-free than AA139-free in the submerged in vitro testing (Table 2), this indicates that the packaging can reduce the toxicity of individual AMPs but does not necessarily equilibrate for individual AMP toxicities. Another possible interpretation of data could be, that the NS packing of M33 did not mitigate toxic effects, since PNP packing did not reduce the LOAEL during submerged in vitro testing, also (Table 2). Using M33-Lip, the combination of M33 in a liposome packaging, a comparable LOAEL to M33-PNP was found for the NS (LOAEL(M33-PNP) 9.0 μg/cm²; LOAEL(M33-Lip) 10.0 μg/cm²) but a clearly smaller LOAEL for the AMP in comparison to M33-PNP (LOAEL(M33-PNP) 1.3 μg/cm²; LOAEL(M33-Lip) 0.3 μg/cm²). On the other hand, toxicity of M33-Lip during ALI testing was clearly reduced in the “high-dose” exposure group (Fig. 6d and e). Hence, it should be taken into account that this NS included the very high molecular ratio of NCAMP of 229:1 and the dose-response relationship (Fig. 6e) resulted in a low toxicity at low dosages but no increased toxicity at higher dosages. So, it cannot be excluded, that these are low-dose effects with a different quality than clear toxic effects such as demonstrated by M33-PNP (Fig. 6d).

To conclude, packing of AMPs into NSs can reduce cytotoxicity as seen with AA139 loading into PNPs or packing into micelles. Furthermore, the results show that the packing does not equilibrate individual toxicity properties of different AMPs as demonstrated by loading of AA139 or M33 into PNPs. Moreover, different packings may have different effects on the toxicity of AMPs as indicated by the differently effective mitigation of M33 toxicity with loading into PNP- or liposome NCs.
4.4. Comparison of effects during submerged testing and overall observations

During submerged in vitro testing, a human alveolar epithelial cell line (A549-cells) was used, which has shown some basic type-II cell characteristics (Lieber et al., 1976; Wu et al., 2017). Due to a relatively large droplet size distribution of the generated aerosol including sizes of smaller than 2 μm and larger than 5 μm (Cossio et al., 2018) using the specific FB-240 nebulizer nozzle, both human bronchial and alveolar lung deposition of the droplets can be assumed. Relevance from the point of regional in vivo deposition of the test items might therefore be given for cells from alveolar origin.

A fundamental difference was found in the LOAELs as identified in the submerged testing system in comparison to LOAELs from ALI testing. Without exception, LOAEL values from submerged testing were significantly higher than from ALI testing. Taking into account the different culture surface of 0.33 cm² and 100 μl medium volume during testing. Without exception, LOAEL values from submerged testing were the submerged testing system in comparison to LOAELs given for cells from alveolar origin.

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Nevertheless, the latter mechanisms can not explain the large differences respect to NS based dosimetry and AMP based dosimetry the LOAEL was reduced by a factor of 4.3. During submerged testing, a reduction of cytotoxicity was also indicated but could not be quantified due to a lack of cellular response within the technically possible dosage range. Packing of AA139 into PNP packs also had a similar effect.

In the case of M33, PNP packing did not reduce toxicity effectively as documented in both test systems, although even the calculation of LOAEL values was mostly impossible from submerged in vitro data due to the lower sensitivity of the system as discussed above. Liposome packing, however, exerted its effect on this AMP as documented by a decreased toxicity in higher exposure doses in ALI testing.

5. Conclusions

An in vitro inhalation model on the basis of ALI cultures of a human lung cell line (A549) was developed to screen for acute toxicity of droplet aerosols. The in vitro test system included a set of control substances (cupper-II-sulfate and lactose) and process controls (concurrent vehicle control exposures and non-exposures) that enabled quantitative dosimetry, indicated significance of results and a given reliability and robustness during experimentation. Moreover, an exposure design was set up to organize the aerosol testing under application of 3 dosage groups on the basis of an E25 and E75 values from the positive control cupper-II-sulfate dose response. In a first application, a set of nanosystems (NSs) including different antimicrobial peptides (AMPs) and nanocarrier (NCs) were tested. In parallel, submerged exposures to test items were conducted as a second source of information on toxic properties of the NSs and characterization of potential different properties of the testing strategies. LOAELs were calculated from dose responses based on NSs and AMPs dosage to enable a collective estimation of results from ALI and submerged in vitro testing.

Packing into nanosystems by nanocarrier reduced the cytotoxicity of AMPs. Particularly, AA139 demonstrated an improvement when packed into NS. M33 took less advantage from the NS, and only from liposomes packaging. LOAELs were significantly higher in the submerged setup compared to the ALI. This was likely to the more relevant ALI exposure scenario including a faster and higher bioavailability of the test items and less protein binding and cross-reaction to the culture media during the 24-h incubation phase before the determination of cellular viability. Therefore, the ALI LOAELs are assumed to be more relevant for the in vivo situation.

In summary, the developed ALI approach successfully enabled in vitro testing of droplet aerosols with a specific given nebulizer in a comprehensive inhalation model and indicated that packing of AMPs into NCs might be a promising way of further development in application of pharmaceutical products for inhalation. Moreover, in accordance to the current state-of-the-art view, it renders that ALI in vitro inhalation models can be promising tools to further develop in vitro methods in the field of inhalation toxicology.

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Declaration of Competing Interest

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