Toxicology in Vitro 63 (2020) 104714

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

In vitro inhalation cytotoxicity testing of therapeutic nanosystems for pulmonary infection

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ARTICLE INFO

Keywords:
In vitro
Inhalation
Nanosystems
Air-liquid interface
Toxicity
Pulmonary infection
Antimicrobial peptides

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

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https://doi.org/10.1016/j.tiv.2019.104714
Received 29 March 2019; Received in revised form 30 October 2019; Accepted 30 October 2019
Available online 06 November 2019

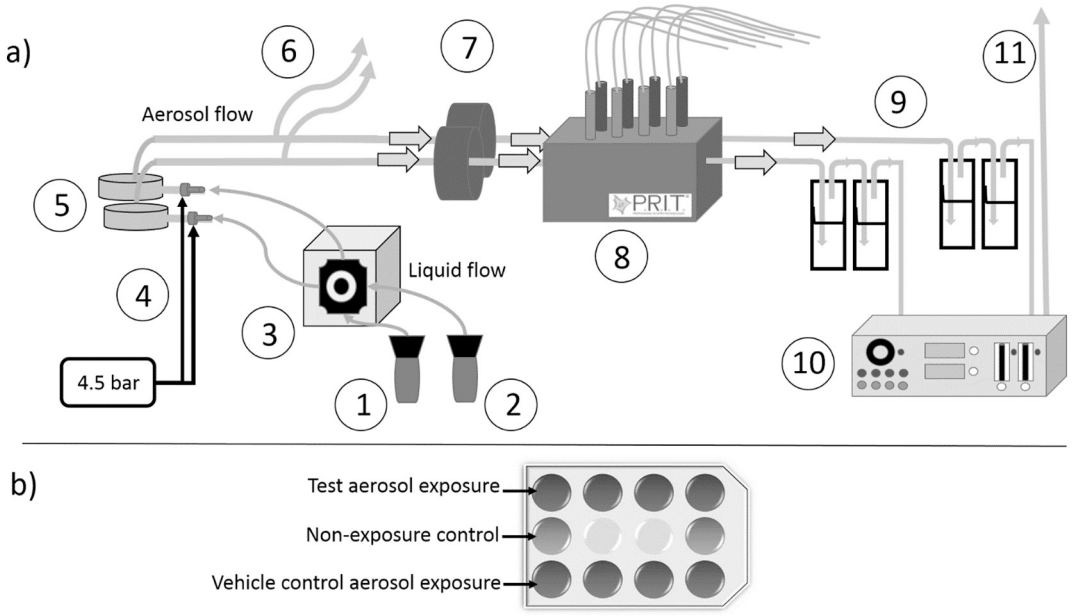

Fig. 1. a) Experimental setup for aerosol generation and air-liquid interface (ALI) cell-based in vitro exposures; (1) reservoir for test substances and positive control substances (2) reservoir for vehicle control (3) peristaltic pump, (4) compressed air (5) nebulizer with glass chamber (Ingeniatrics, 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.

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/cm^2], 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; Miyoung et al., 2015; Tsaioun 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 (Cossío 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 2.9 μm with 64 to 75% between

0.9 and 5.25 µm as determined by laser diffraction and a completely decayed 2-deoxy-2-(18F)fluoro-D-glucose tracer (Cossío 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.® ExpoCube®, Ritter and Knebel, 2014). It was positioned in-line to the aerosol generation (Fig. 1) and enabled a concurrent test aerosol exposure, 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 applicability under the test conditions (water solvability, usability in nebulizer, analytical accessibility for deposition measurements) lactose (Sigma-Aldrich, Germany, L3750) and copper-II-sulfate (Roth, Germany, CP86.2) were selected as negative and positive controls, respectively.

For the submerged exposure, a "toxic polymer" (poly(2-dimethylaminoethyl 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 aerosol / ALI testing and using a conventional submerged in vitro approach. AA139 was combined with PNPs and micelles (AA139-PNP, AA139-Mic) and M33 was combined with PNPs and loaded into a lipid nanosystem (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 conjugated polyethylene glycol (DSPE-PEG2000). The lipid nanocompound in the "M33-Lip" NS consisted of a 10:1:0.6 mixture of egg phosphatidylcholine (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 routinely cultured in 75 cm^2 flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Biochrom GmbH, Germany) and 0.01% Gentamicin at 37 °C in a humidified atmosphere containing 5% CO_2. For cytotoxicity testing, subconfluent 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 8×10^3 per well. Prior to treatment with test items, cells were allowed to proliferate to reach 70–80% confluence. Before exposure, culture medium was changed to DMEM without fetal calf serum. Test items were added and a tetrazolium salt cleavage based cell viability assay (WST-1) was performed after 4 h and 24 h.

For air-liquid interface culture an aliquot of $4x10^4$A549 cells was seeded on microporous membranes (Inserts, BD Falcon; 0.4 µm pore size; growth area ~1 cm^2). Cells were further cultivated on the membranes 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 exposure with the test substances, residual liquid from the apical side of each cell monolayer was gently removed. During the treatment, cells were nutrified 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 schematically 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 cytotoxicity. 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 tetrazolium salt conversion was carried out. Therefore, cells were incubated for 60 min at 37 °C with 500 µl of medium from the apical side containing 10% WST-1 solution per membrane. Aliquots of 100 µl were transferred into 96-well microplates for measuring their absorbance at 450 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 exposure, the culture membranes were rinsed with 200 µl water and these samples were analyzed using a cupper-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	NS composition for aerosol / ALI testing		Test items for submerged testing	
	AMP	NC	NS	NS
	conc. [mg/ml]	conc. [mg/ml]	conc. [mg/ml]	Composition
AA139-free	12.70	–	–	Dilutions from pure AA139
AA139-Mic	12.60	34.00	46.60	Dilutions from NS (10.4 mg AA139 / 32.26 mg micelles)
AA139-PNP	4.00	40.00	44.00	Dilutions from NS (0.1 mg AA139 / 1 mg PNP)
M33-free	- *)	–	–	Dilutions from pure M33
M33-PNP	5.60	40.00	45.60	Dilutions from NS (0.14 mg M33 / 1 mg PNP)
M33-Lip	1.70	58.30	60.00	Dilutions from NS (3.2 mg M33 / 83.26 mg lipids)

*) 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% cupper-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/cm^2 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 from 0.9% NaCl) did not induce any significant effects in the viability of the cells compared to non-exposure controls.

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 EC_{50} value of 11.4 µg/cm^2 with a lowest observed adverse effect level (LOAEL) of 4.6 µg/cm^2. In contrast, exposure to lactose aerosols did not induce significant toxicity up to highest dosages of 90 µg/cm^2.

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 EC_{75}- and EC_{25}-values (6.5 and 17 µg/cm^2, respectively) from the respective dose response relationship as depicted in Fig. 5. The dosage in the highest dosage group (dosage ≥17 µg/cm^2) 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/cm^2 (Fig. 6a).

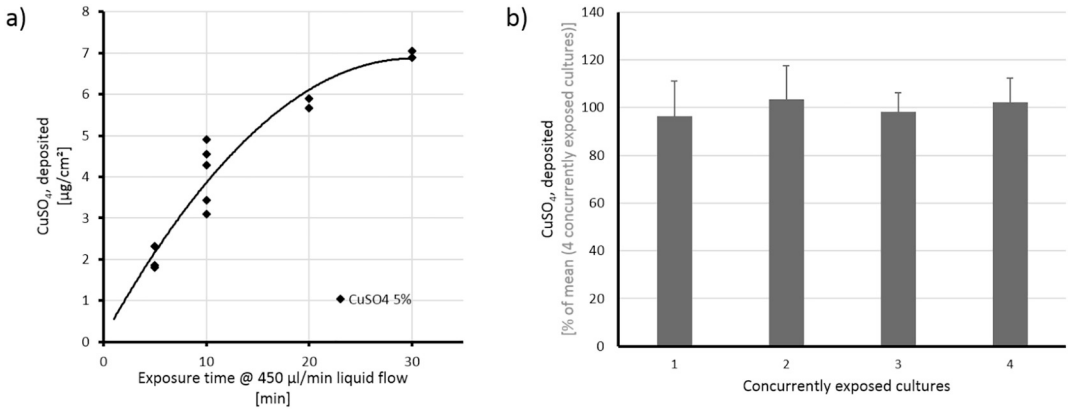

Fig. 2. Determination of deposited dose and relative deposition efficiency in the exposure device. a) Results of measurements of the delivered dose on microporous membranes in the cellular exposure situation with copper-II-sulfate aerosols depending on the exposure time. Dots represent results from independent 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.

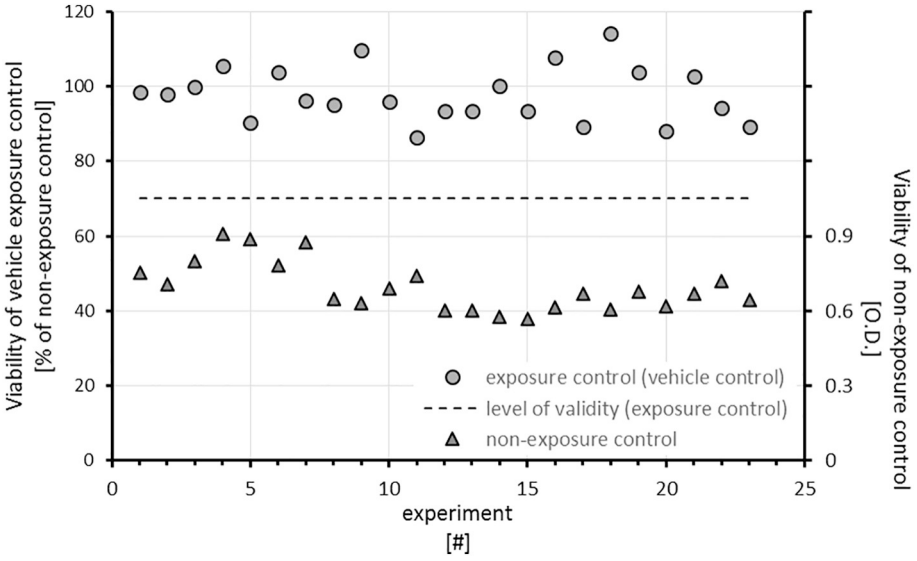

Fig. 3. Representative results from vehicle exposure controls and non-exposure controls from a series of 23 independent exposure experiments. Triangles (right y-axis) represent WST-1 raw data (optical densities) as a mean of 2 technical replicates of non-exposure controls from single experiments. Circles (left axis) represent the viability of the exposure controls / vehicle exposure controls of the 4 technical replicates as %age of control values referenced to the non-exposure controls. The dotted line at 70% viability of controls represents the level of validity for each single experiment based on the viability of the exposure controls.

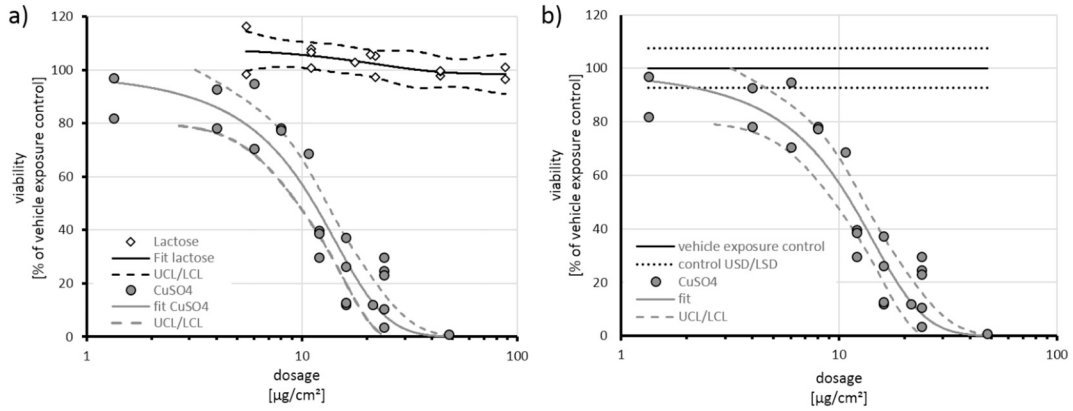

Fig. 4. Results from exposures of A549 human lung cells to control aerosols. a) Droplet aerosols from copper-II-sulfate (positive control) ($CuSO_4$) and lactose aerosol (negative control) with fitting and upper and lower confidence limits (95%) resulting in an EC_{50} value for $CuSO_4$ of 11.4–1.8/ + 1.7 µg/cm^2. b) Estimation of lowest observed adverse effect level (LOAEL) from cupper-sulfate data by calculation of the variability of the controls (grey line with one standard deviation (grey dotted lines)) and comparison to dose response fitting with variability (black line with 95% confidence intervals (black dotted lines)) resulted in a LOAEL of 4.6 µg/cm^2.

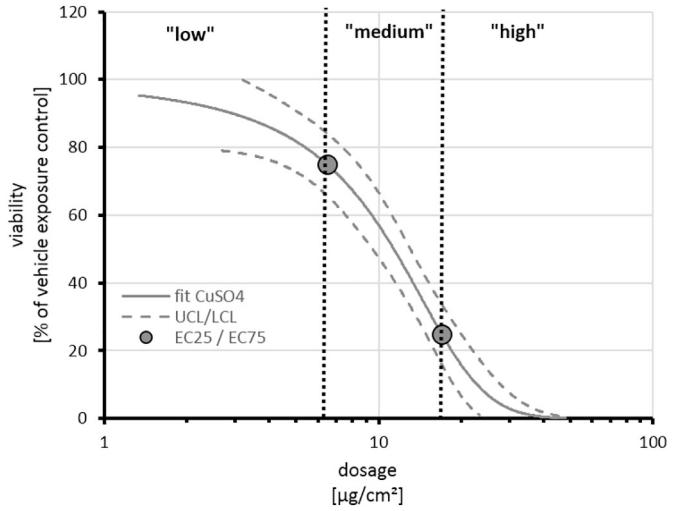

Fig. 5. Evaluation of results from exposures to the positive control copper-II-sulfate to determine exposure group dosages for test items based on calculation of EC_{75} and EC_{25} values at 6.5 µg/cm^2 and 17 µg/cm^2, respectively.

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/cm^2 (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/cm^2, 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/cm^2 (Fig. 6d). The LOAEL was calculated as 9.0 µg/cm^2.

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/cm^2 and higher and, hence, induced a clearly lower toxicity than the positive control cupper-II-sulfate (Fig. 6e).

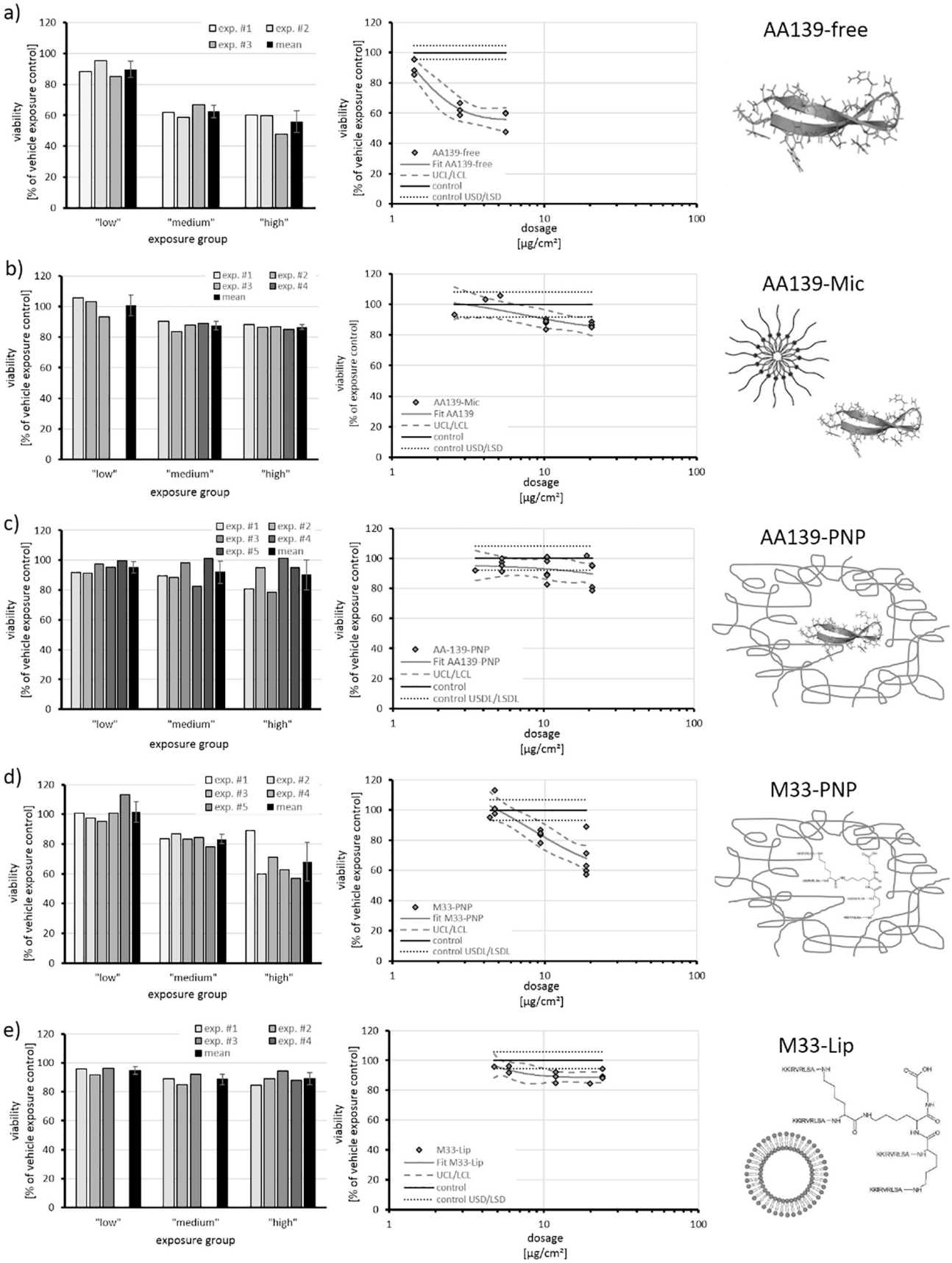

Fig. 6. Results from exposures of human lung A549 cells towards aerosols generated from the test items. Left: Results from single experiments based on exposure groups low, medium or high, middle: Dose response relationships based on the calculation of the delivered dose and variability of controls for determination of LOAELs; right: structures of the test items. PNP structures have only illustrative character (c, d).

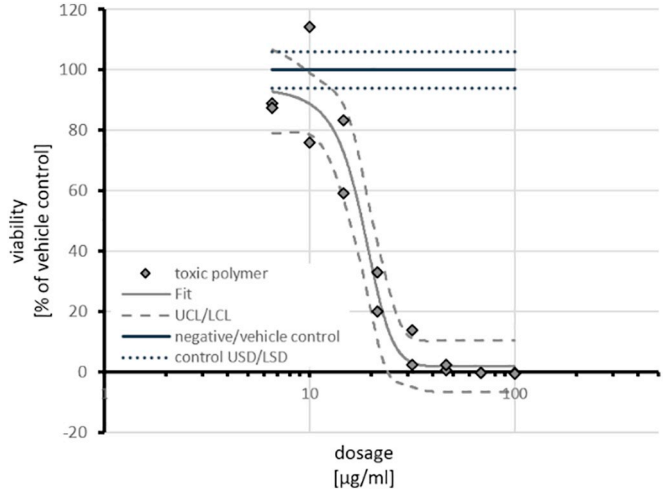

Fig. 7. Application of the toxic polymer as a positive control during submerged in vitro testing. Dose response using A549-cells with fitting, confidence intervals (95%) and variability of controls for LOAEL estimation.

3.2.2. Standard submerged in vitro exposure setup

3.2.2.1. Positive control "toxic polymer". A549 cells were exposed to varying dosages of the toxic polymer under standard submerged culture conditions in a 96-well microplate exposure setup. Fig. 7 displays the resulting viability related dose response fitting, confidence levels (95%) and variations of controls for determination of LOAELs (12.7 µg/ml).

3.2.2.2. Submerged testing of AMPs and NSs. According to the testing of the toxic polymer, AA139-free, M33-free and their combinations with NCs to NSs were tested in the submerged assay. Table 2 shows a compilation of all LOAELs together from submerged and ALI in vitro toxicity testing.

4. Discussion

The overall aim of this study was the establishment of an experimental setup for in vitro inhalation testing under application of a present aerosol generation device and conduction of a first series of tests with a group of specific test items including nano-systems with antimicrobial 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 PADDOCC system (Hein et al., 2010) a commercial dry-powder inhaler, 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 present 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 inhalable 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 investigations focused on (1) the technical (droplet deposition) and biological (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 nebulizer system (Ingeniatrics; Cossío et al., 2018) and characterized with respect to droplet deposition (Fig. 2), by use of aerosolized $CuSO_4$ solutions. 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

Table 2
LOAELs from ALI and submerged in vitro testing including dedicated controls for the different test setups; results from application of the testing procedures to the AMP AA139 and packaging into micelle based or polymeric nanoparticles (PNPs) and results from application of the testing procedures to the AMP M33 and packaging into PNPs and liposome based nanosystems.

Test item	Remark	LOAELs based on NS dosage			LOAELs based on TEST ITEM dosage		
		Submerged testing		ALI testing	Submerged testing		ALI testing
		A549		A549	A549		A549
		4 h	24 h	24 h [µg/cm²]	4 h	24 h	24 h [µg/cm²]
Lactose	negative control for ALI testing						> **88**
Cupper-II-sulfate	positive control for ALI testing						**4.6**
Toxic polymer	positive control for submerged testing					13	
AA139-free	AMP without NC packaging	*978*	*464*	***3.9***	315	150	**1.4**
AA139-Mic	AMP AA139 in micelle packaging	> 2051	> 2051	**17**	> *500*	> *500*	***6.2***
AA139-PNP	AMP AA139 in PNP packaging	> 550	> 550	> **21**	> *50*	> *50*	> ***2.1***
M33-free	AMP without NC packaging				94	64	
M33-PNP	AMP M33 in PNP packaging	> 570	> 570	**9.0**	> *70*	> *70*	***1.3***
M33-Lip	AMP M33 in liposome packaging	8624	6811	**10**	*319*	*252*	***0.3***

Standard typo tested item. *Italics* typo data calculated from tested item

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/cm^2 cupper-II-sulfate were deposited on the cellular surface per minute using the system during aerosolization of 5% cupper-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 cupper-II-sulfate as a positive control the responsiveness of the in vitro inhalation model towards human inhalable toxicants could be shown. The EC_{50} value for cupper-II-sulfate exposure of A549 cells was detected at 11.34 μg/cm^2 (Fig. 4). In an earlier study, where cupper-II-sulfate was also applied from an airborne state, but as a dry particle aerosol and not a droplet aerosol, an EC_{50}-value of 23.66 μg/cm^2 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, solvability, 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 cupper-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 EC_{50}-value of $CuSO_4$. During submerged testing, 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/cm^2, LOAEL(AA139-Mic, NS) = 17 μg/cm^2, LOAEL(AA139-free, AMP) = 1.4 μg/cm^2, LOAEL (AA139-Mic, AMP) = 6.2 μg/cm^2). 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/cm^2; LOAEL(M33-Lip) 10.0 μg/cm^2) but a clearly smaller LOAEL for the AMP in compare to M33-PNP (LOAEL(M33-PNP) 1.3 μg/cm^2; LOAEL(M33-Lip) 0.3 μg/cm^2). 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 NC:AMP 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 (Cossío 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 cm^2 and 100 µl medium volume during submerged and 1 cm^2 during ALI testing and converting the concentration-based submerged LOAELs [µg/ml] into a surface-load [µg/cm^2], a formal conversion factor of 0.33 is generated for calculation of submerged LOAELs in a surface-load based dimension. In the case of AA139-free the submerged LOAEL of 150 µg/ml would therefore correspond to a "submerged surface based" LOAEL of 45.0 µg/cm^2. This calculation is based on the assumption that the complete amount of test substance dissolved and present in the culture media during submerged cell exposure is in continuous contact to the cellular surface.

However, the actual delivered dose under submerged conditions may be substantially lower than that. Physico-chemical based deposition mechanisms such as sedimentation and diffusion are depending on parameters such as particle or agglomerate sizes and result in transfer rates from solution to the cellular surface that might clearly reduce the actual surface dose. Computational models such as the ISDD model (Cohen et al., 2014, Hinderliter et al. 2010, DeLoid et al. 2015) enable a theoretical prediction of the actual delivered dose taking into account particle characteristics such as density and size and physico-chemical conditions such as time, temperature, media viscosity and well-known theoretical relationships such as diffusion or sedimentation velocity. On the basis of these models, titanium dioxide micro or nanoparticles as model substances would result in particle size dependent transfer rates resulting in deposited fractions of 0.5 to 1 for a 24 h exposure time, meaning that at least 50% (30 or 50 nm particles) of the particles present in the culture actually get in contact to the cellular surfaces. The lowest transfer rate published on the base of this model (Cohen et al., 2014) is 0.0101 h^{-1} for carbon nanohorns (20.3 nm primary particle size) resulting in a 24% deposition rate for 24 h.

The large difference of the LOAELs between submerged (45.0 µg/cm^2) and ALI (1.4 µg/cm^2) exposure, however, might indicate losses of the test item in the submerged test system due to protein binding in culture media or plastic surfaces, lower test item stability during dissolution in culture media, other kinetic events such as transport in and out of the cells (Hamon et al., 2015) or the relevance of transport rates such as discussed above. Nevertheless, the latter mechanisms can not explain the large differences between submerged and ALI LOAELs alone. Taking into account the lowest deposition rate of 24% (see above), the submerged LOAEL for the test item AA139-free would result in an effective LOAEL of 10.8 µg/cm^2, which is still factor 7.8 higher as the respective ALI value (1.4 µg/cm^2). This is even more extreme for the test item M33-Lip with an effective submerged LOAEL of 18.4 µg/cm^2 in compare to an ALI LOAEL of 0.3 µg/cm^2 (factor 61.). More likely, the in vivo relevant and immediate contact between the deposited aerosol droplets and the cellular surface during ALI exposure might lead to a higher and faster bioavailability of the test items and might therefore result in a more realistic estimation of the LOAEL during ALI testing in comparison to the submerged testing scenario.

Consistently in both in vitro testing strategies and depending on the nature of AMPs and NSs, packing of AMPs into NSs reduced the cytotoxicity. This was demonstrated very clearly by comparison of AA139-free and AA139 packed into micelles (AA139-MIC) in ALI testing. With 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 PNPs 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 EC_{25} and EC_{75} 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.

Funding

PneumoNP has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement no 604434.

Declaration of Competing Interest

All authors received a grant from EU, 7th Framewok Programme, grant #604434, for the conduct of the study. D.R., J.K., M.N. and T.H., are employees of the Fraunhofer Gesellschaft, Germany, a patentee of the ExpoCube® Technology (DE 102013 109,450). I.L., M.M. and R.G. have a patent on a process for preparing water dispersable polymer nanoparticles licensed. C.F. and A.P. have a relevant patent licensed. MS is an employee and shareholder of Adenium Biotech ApS, which owns the patent of AA139.

Acknowledgements

The authors thank Ines Voepel, Martin Engelke and Andreas Hiemisch for their excellent technical assistance.

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