Cytotoxicity testing of therapeutic nanosystems for pulmonary infection using an air-lifted interface in-vitro test system

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Introduction

Infections caused by antibiotic resistant bacteria are one of the current major threats affecting global health, largely due to a lack of effective antibiotic therapies. To combat this problem new nanocompounds are developed as drug carriers for combined antibiotic systems. For respiratory infections the application of these antibiotic nanosystems (NS) as an aerosol in the lung is the first choice.

Objectives

The aim of the study was to investigate the cytotoxicity of four selected airborne NS and a free antimicrobial peptide compared to reference control substances in a cell-based in vitro model. Therefore a realistic in vitro test environment was established including (1) a nebulizer (Ingeniatrics FB-240) for generating a droplet aerosol (2) an exposure device (P.R.I.T.® ExpoCube®) for exposure of cells as well as (3) a human lung cell line (A549) grown and exposed under air-lifted interface conditions on semipermeable membranes.

Materials and Methods

Cellular Model system: The A549 human lung cell line was purchased from a commercial supplier (ATCC; LGC Promochem). Cells were routinely taken from a stock pool and grown in 75cm2 flasks by use of Dulbecco´s MEM medium (Seromed, Berlin) supplemented with 10% FCS and antibiotics. To mimic the exposure situation of the epithelium in the in vivo lung the air-liquid-interface (ALI) technique based on cell cultures on microporous membranes was used. Therefore, cells were initially cultivated under their cell type specific conditions in 75cm² culture flasks using submerged conditions. Culture medium was changed every to 2-3 days. Before reaching 80% confluence, cells were subcultivated. During a cell passage an aliquot of the cells was then seeded ($4x10^4$) on microporous membranes (Inserts, BD Falcon; 0.4µm pore size; growth area ~1cm²). Cells were further cultivated on the membranes for approximately 72hrs until they reached a confluent monolayer as inspected by light microscopy. Serum was removed from the culture during a medium change 16-18hrs before exposure. Previous 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 solely while being exposed to the test and reference (control) substances from the top .

Test items, reference items and vehicle controls: Four combined nanosystems (substance # 1- #4) were obtained from the project partners University of Utrecht and CIDETEC. The free antimicrobial peptide (substance #5) was purchased from the project partner Adenium. For control purposes, cells were exposed in parallel to sodium chloride (0.9%) solution or HBS buffer which served as relevant negative (vehicle) controls in each experiment. $CuSO_4$ (copper-II-sulfate) served as a positive control, lactose as a negative control. They were aerosolized from an aqueous solution and applied as a droplet aerosol to the lung cells.

Cell Exposure: Air–lifted interface cultures from A549 cells were exposed using the P.R.I.T.® ExpoCube® (figure 1). Aerosols from test-, positive or negative substances were generated by nebulization using the Ingeniatrics Nebulizer (FB-240). Control exposures included concurring sodium chloride (0.9%) or HBS buffer exposures during each single experiment.

WST-1 assay: Cells were analyzed with regard to viability (WST-1) after a 24hours post-exposure re-incubation period under cell-specific conditions inside an incubator.

Dosimetry: For the calculation of the individual dose of each individual experiment, the deposition of the droplets on the culture membranes under the conditions of the exposure was investigated by experiments using the positive substance $CuSO_4$ and a spectrometric analyst method.

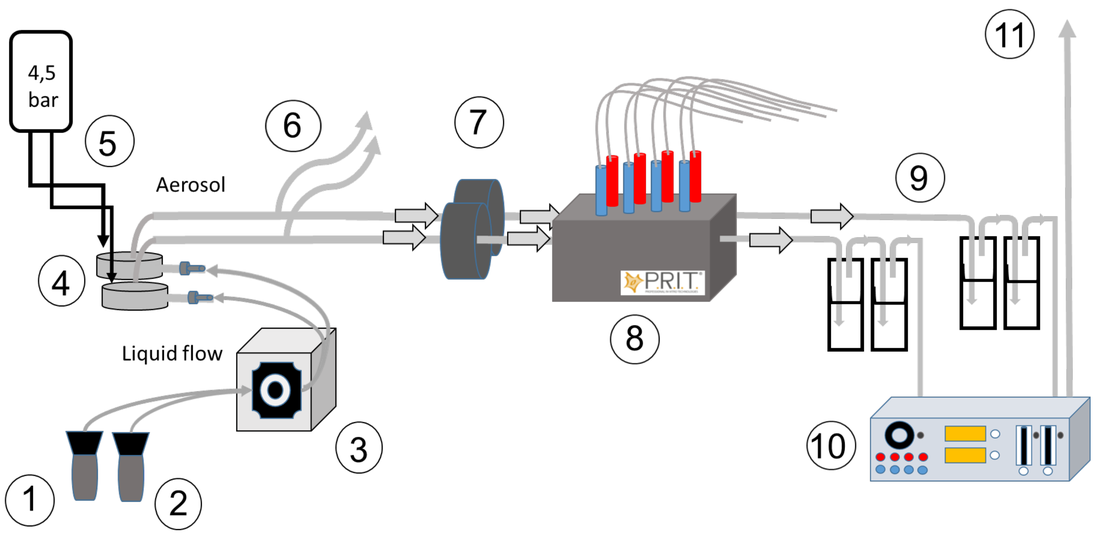

Figure 1: Experimental setup of aerosol generation and air-lifted interface exposures, with (1) reservoir for test substances and positive control substances (2) reservoir for negative control substance (3) peristaltic pump (4) nebuliser with glas chamber (Ingeniatrics), (5) compressed air (6) excess aerosol, (7) photometer, (8) P.R.I.T.® ExpoCube® (9) Impinger (10) P.R.I.T.® ControlUnit (11) waste.

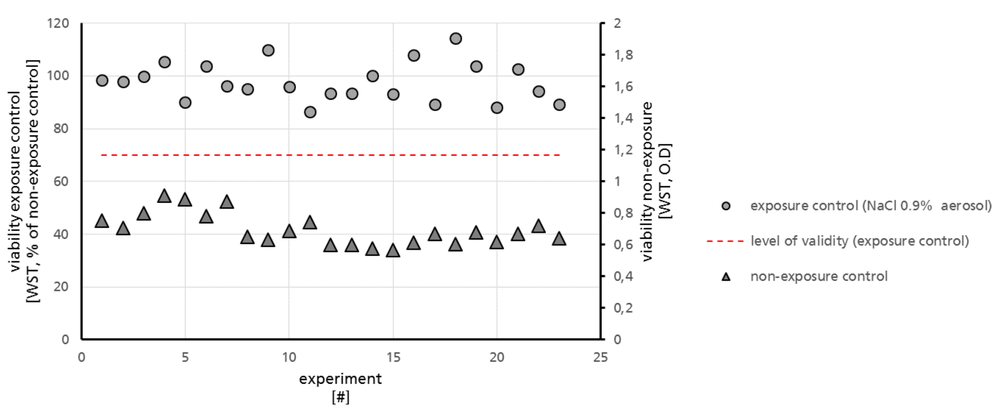

Figure 2: Representative results for the exposure to the vehicle control aerosol (droplets composed of sodium chloride 0.9% solution) (dots) and the unexposed control cells (triangles).

Results

- No significant adverse effects on the cells by the exposure scenario as indicated by the vehicle controls/exposure controls (Figure 2).
- Positive control copper-II-sulfate demonstrates that the model is sensitive to acute toxic effects induced by aerosols (Figure 3A). Comparison to results from negative control lactose indicates a significance and predictivity of the test system with respect to detection of toxicity (Figure 3A).
- Free antimicrobial testsubstance revealed highest toxicity compared to the positive control Figure 3B - F)
- Packing the antimicrobial testsubstance into a nanocoumpound can significantly reduce its toxicity (Figure 4A)
- Upon the nanosystems, NS#2 exhibited a significantly higher toxicity in the highest dosage (based on nanosystem dosage) (Figure 3D,4B).

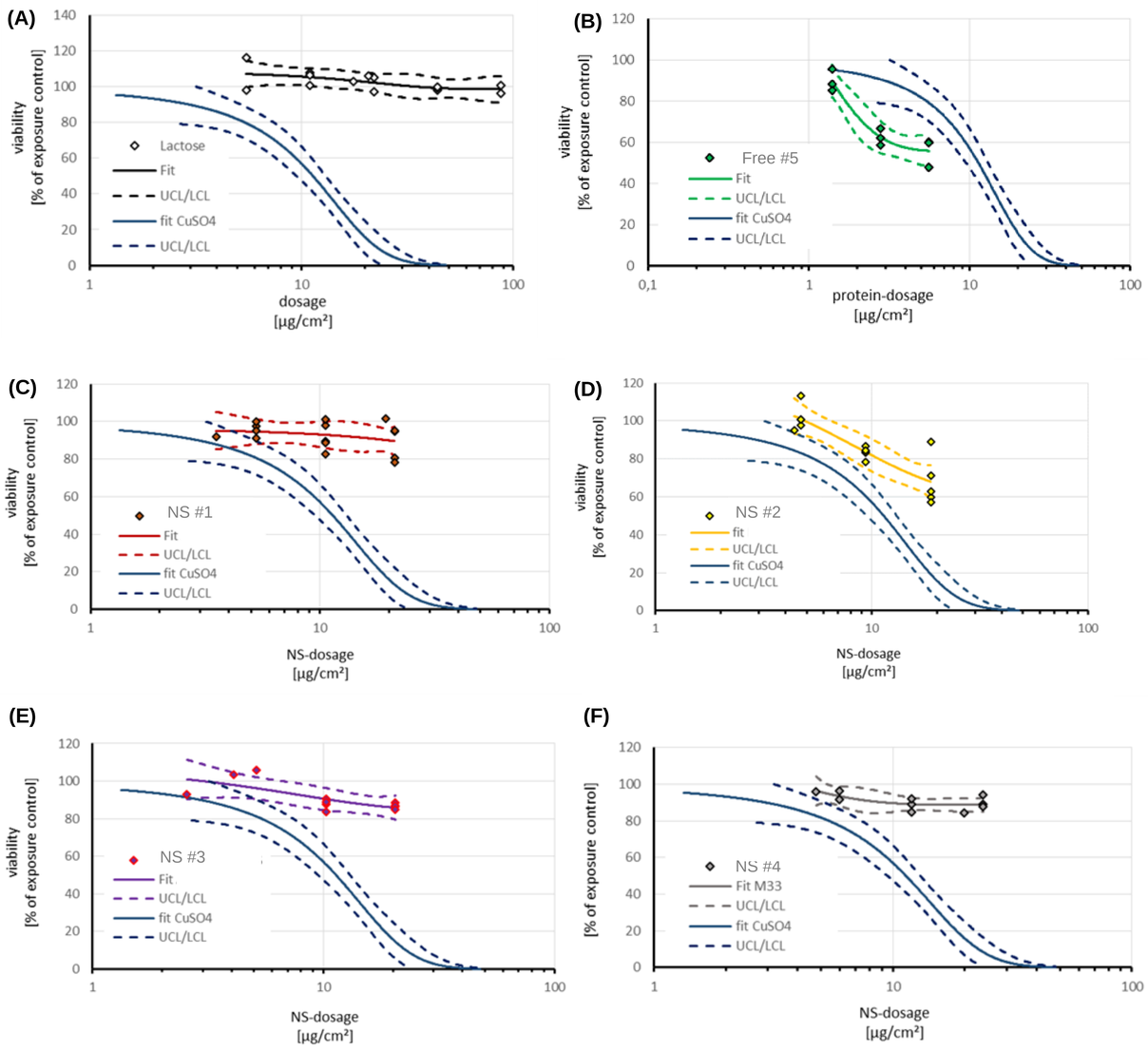

Figure 3 : Dose effect curves. (A) negative control aerosol (lactose) compared to the positive control aerosol (copper-II-sulfate). (B) free antimicrobial testsubstance (#5) compared to the positive substance $CuSO_4$. (C - F) nanosystem #1 to nanosystem #4 compared to the positive substance $CuSO_4$.

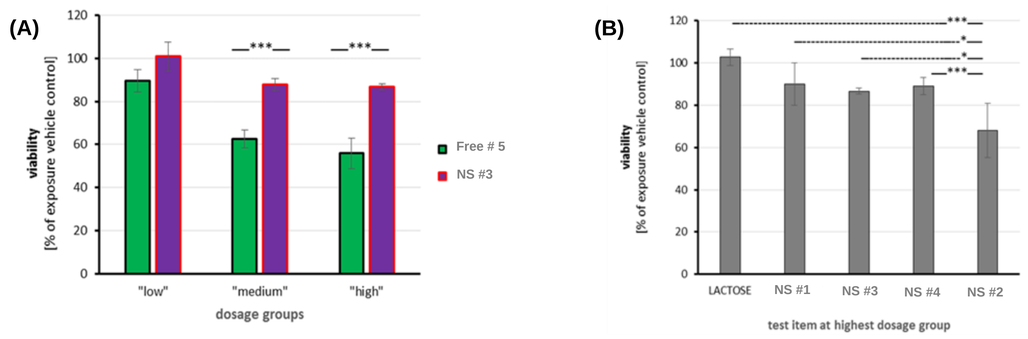

Figure 4 : (A) Free antimicrobial peptide compared to its corresponding nanosystem on dosage based grouping (same protein in dosage). Packing into specific nanocompounds significantly reduced the toxicity of the antimicrobial peptide. (B) Impact on the cell viability dependent on the nanosystems at highest dosage. NS#2 shows significantly the highest effect.

Conclusions

- To mimic the in-vivo situation, the cells of a lung epithelial cell line are exposed to the testsubstances under investigation at the air-liquid interface by using the Fraunhofer-patented P.R.I.T.® technology.
- Tests can be performed under close to real-life conditions, applying the antibiotic nanosystems in the form of aerosols.
- Packing into nanosystems can significantly reduce toxicity of the antimicrobial compound.
- Efficiency of the protection might be dependent on antimicrobial peptide / nanocompund combination.
- The described method allows potentially toxic candidates to be identified and discarded already at an early stage of drug development.

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