Validation of the ALICE-CLOUD technology for functional efficacy studies with aerosolized drugs delivered to cells at the air-liquid interface

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Summary

Background. Aerosol-based inhalation therapy is widely used for the treatment of lung diseases, but reliable drug screening approaches in complex phenotypic settings (e.g. cell systems) at the air-liquid interface (ALI) are unavailable. Here, we introduce the ALICE-CLOUD technology, which utilizes the principles of cloud motion for delivery of bioactive aerosolized liquid drugs to pulmonary cell types cultured under physiologically realistic ALI conditions.

Methods. Drug-to-cell delivery efficiency of the ALICE-CLOUD was investigated with fluoresceine as surrogate drug. A novel candidate drug for anti-inflammatory inhalation therapy Bortezomib (Velcade®) was investigated by stimulating human alveolar epithelial cells (A549) with TNFα resulting in a 7-8-fold activation of the IL-8 promoter. Bortezomib (Velcade®) was applied to the cells and the effect on IL-8 promotor activation and proteasome activity was investigated in cell lysates using a luciferase reporter assays. Cytotoxicity was monitored with the WST-1 and LDH assays.

Results. For 200 µL of nebulized liquid, 33.6 µL was delivered within 3.5 min to 6-well transwell inserts (5.6 µL per insert) corresponding to a 16.8% delivery efficiency. The reproducibility of the dose was 9.3% and the insert-insert variability was 4.3%. We found that Bortezomib (Velcade®) can be aerosolized to efficiently block proteasomal activity and mediate potent anti-inflammatory effects in A549 cells cultured at ALI conditions. Importantly, aerosolized and liquid (non-aerosolized) drug delivery showed identical drug efficacy. Of note, the response kinetics of aerosolized Bortezomib was by about a factor of 12 faster than non-aerosolized Bortezomib delivered under submerged conditions.

Conclusion: Our data validate the ALICE-CLOUD as an easy-to-handle, quantitative, and highly suitable tool for preclinical screening for inhalation drugs at realistic conditions. Moreover, we identify Bortezomib – and possibly also other representatives of this class of biopharmaceutics - as a promising candidate for inhalation therapy.

Introduction

Traditionally, preclinical *in vitro* drug screening relies on dissolution of drugs in cell culture medium and application of drugs to submerged cell cultures [1, 2]. For the lung, aerosolized drug delivery to cells cultured at the air-liquid interface (ALI) represents a much more realistic exposure scenario potentially allowing for better predictive power of the *in vitro* screening results for the clinical outcome. While realistic ALI models of the pulmonary tissue barrier are available [3], currently available aerosol-to-cell delivery systems are either not optimized for dose-controlled, efficient and spatially uniform drug delivery and/or experimentally difficult to handle.

Here, the ALICE-CLOUD technology for therapeutic aerosol is introduced (ALICE: **A**ir-**L**iquid **I**nterface **C**ell **E**xposure), which utilizes principles of cloud motion (CLOUD) for efficient and bioactive delivery of aerosolized liquid drugs to ALI pulmonary cells. The drug delivery performance of the ALICE-CLOUD was experimentally determined. The functional efficacy of a novel candidate drug for inhalation therapy (the proteasome inhibitor Bortezomib) was investigated with the ALICE-CLOUD and finally, the results were compared to the conventional method for drug efficacy testing (non-aerosolized delivery, submerged cell culture).

Methods and Materials

We have previously used cloud-based delivery of liquid aerosols to cells for dose-controlled delivery of toxicologically relevant materials [4,5]. However, screening for novel drug candidates has to meet more stringent requirements regarding exposure time, automation-friendliness and substance efficiency, which have been accommodated in the ALICE-CLOUD technology introduced here.

The ALICE-CLOUD consists of a cuboidal exposure chamber large enough for housing a standard multi-well plate at the bottom of the chamber. All parts can easily be disassembled and sterilized. Aerosol-to-cell delivery with the ALICE-CLOUD was performed with a vibrating mesh nebulizer (Aeroneb Pro, Aerogen Inc., Galway, Ireland) positioned at the top of the chamber. The nebulizer ejects 200 µL of a liquid in form of a narrow, dense cloud of droplets (ca. 4-6 µm droplet diameter) with an output rate of about 0.4 mL/min and an initial speed of about 2 m/s vertically downwards towards the multi-well plate. Near the bottom of the chamber, the cloud is diverted horizontally into all directions and then vertically up at the lateral walls. This results in formation of a vortex, which converts the spatially well-defined cloud into a uniformly distributed mist gradually filling the entire chamber. The uniformly distributed mist settles gently within ca. 3.5 min onto the cells.

Characterization of drug delivery efficiency: The drug delivery performance of the ALICE-CLOUD was determined by nebulizing 200 µL of fluorescein-PBS solution (15 µg/mL). The amount of fluorescein collected in each of the 6-well transwell inserts (used for cell culturing) was quantified by fluorescence analysis (ex/em: 483/525 nm).

Design of the drug efficacy study: As a proof-of-concept study for functional drug screening the therapeutic efficacy of a novel candidate drug for inhalation therapy, the proteasome inhibitor Bortezomib (Velcade™), was investigated. Human lung cells were stimulated with tumor necrosis factor α (TNFα) to induce a 7-8 fold activation of the IL-8 promoter. The therapeutic efficacy of Bortezomib was assessed by measuring the dose-dependent inhibition of IL-8 promoter. For further validation of the ALICE-CLOUD, we also performed comparative measurements with non-aerosolized Bortezomib applied to A549 cells at submerged culture conditions. Importantly, A549 cells were chosen for their lack of polarization at ALI conditions, which eliminates potential inadvertent differences in cellular response due to different cell culture conditions.

The proteasome inhibitor Bortezomib: Bortezomib (Velcade®), an FDA-approved proteasome inhibitor for systemic treatment of multiple myeloma, is known to have anti-inflammatory effects [7]. . 200 µL of serial dilutions of Bortezomib (10 to 400 µM) were nebulized with the ALICE-CLOUD. 3 µl of a 0.9% NaCl solution was added to each 250 µL batch of solution to provide enough ionic strength for reliable operation of the nebulizer.

Cell handling and IL-8 assay: All exposure experiments were performed with a human alveolar epithelial-like cell line (A549) from a lung adenocarcinoma (obtained from ATTC, Manassas, VA, USA) [8]. A549 cells were seeded into cell culture inserts and initially cultivated at submerged cell culture conditions using DMEM/F12/L-Glut/15 mM HEPES buffered medium (Invitrogen, Germany) containing 100 Unit/mL penicillin, 100 µg/mL streptomycin, 0.5 mg/mL gentamycin sulfate and 10 % fetal calf serum. Cells were grown to confluence (takes 4 days) transferred to air-liquid interface conditions (for 24h) and exposed to TNFα and Bortezomib.

For submerged experiments, cell handling was identical as for ALICE-CLOUD experiments except for not transferring the cells to ALI conditions 24 h prior to treatment.

Cellular response analysis: Morphology of the cells was investigated with confocal microscopy. The use of IL-8 luciferase reporter cells (A549; courtesy of A. Duschl, Univ. Salzburg, Austria; [6]) allowed monitoring of the IL-8 promoter activity with a luciferase assay. Proteasome activities were measured in cell lysate using the Proteasome-GloTM cell based reagents for chymotrypsin-like, trypsin-like and caspase-like activities according to the manufacturer's instructions (Promega, Cat. No. G8531, Mannheim, Germany). The observed luciferase activity was normalized to the protein concentration to account for difference in cell number. Cell viability and cytotoxicity were assessed using the WST-1 and LDH assay, respectively.

Results

Drug delivery performance of the ALICE-CLOUD: For 18 independent nebulizations of 200 µL of fluorescein a mean volume of 5.6 µl per transwell insert (6-well size; 13.3µm layer thickness) was detected corresponding to a drug-to-cell delivery efficiency of 16.8%. The repeatability of the mean dose was ±9.3% (1σ) and the mean insert-insert dose variability was 4.3% (1σ) (Figure 1, left). Considering the limited cell-coverage of 20.2 % for a standard 6-well multi-well plate (with inserts), this implies that 83.2% (16.8%/20.2%) of the invested substance (200 µL) is deposited on the bottom plate of the ALICE-CLOUD. Only one of the inserts showed a minor systematic mean error of -3.9% outside the statistically expected 95% confidence interval of 2.1%. 95% of the

final cell-deposited dose is delivered within less than 2 min. Hence, for 200 µL of nebulized liquid, we conservatively recommend a total exposure time of 3.5 min which corresponds to a net drug delivery rate of 0.4 µL/cm^2/min (= 5.6 µL/4.2 cm^2/3.5 min).

Efficacy of aerosolized Bortezomib using the ALICE-CLOUD: The ALICE-CLOUD exposure itself did not have any adverse effect on IL-8 promoter activity or cytotoxicity. ALICE-CLOUD mediated delivery of aerosolized Bortezomib to A549 cells at ALI conditions resulted in a dose-dependent inhibition of TNFα-induced IL-8 promoter activation at non-toxic doses starting from 50 µM. Of note, efficient inhibition of IL-8 promoter activation was only achieved when the chymotrypsin-like and caspase-like proteasome activity was inhibited by more than 90% by aerosolized Bortezomib, which is in agreement with the known pharmacological profile of Bortezomib.

Efficacy of non-aerosolized Bortezomib: For comparison, we assessed the therapeutic efficacy of non-nebulized Bortezomib at submerged cell culture conditions, where cells were treated identically to ALI conditions, except for not transitioning them to ALI conditions. Confocal microscopy revealed that cells formed a tightly packed, confluent layer on the perforated membrane of the transwell inserts at both culture conditions. All findings reported for ALI conditions were matched, except that the dose-dependent inhibition of IL-8 promoter activation occurred at lower molar doses (above 2.5 µM).

Effect of aerosolization on drug efficacy: On first sight, these data suggest that submerged cell culture conditions are far more sensitive than ALI conditions, as the equipotent dose of Bortezomib that induced a significant reduction of the TNFα-induced IL-8 promoter activation is by a factor of about 25 lower for submerged conditions (50 vs. 2.5 µM). As nebulization itself could have an effect on the efficacy of Bortezomib, we compared the efficacy of aerosolized versus non-aerosolized Bortezomib using a cell-free (purified 20S proteasome) and a cell-based assay (A549). No degradation of the functional activity of Bortezomib due to nebulization with an Aeroneb Pro nebulizer was observed.

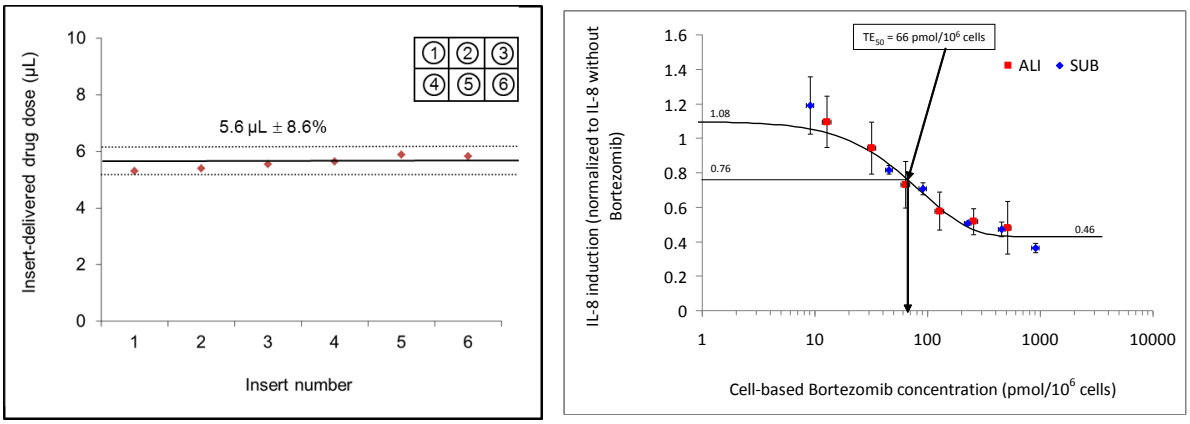

Figure 1: **Insert-delivered drug dose and comparative functional drug efficacy (Bortezomib) for submerged and ALI conditions**. *Left*: Drug delivery characteristics of ALICE-CLOUD measured with 200 µL fluorescein-PBS solution as surrogate drug. On average 5.6 µL of drug (±8.6% (2σ); n = 18) is delivered to each of the 6 transwell inserts corresponding to a 16.9% drug-to-cell delivery efficiency. *Right*: Bortezomib reduces IL-8 promoter activation in TNFα-challenged A549 cells and 50% of the maximum therapeutic efficacy (TE_{50}) is reached at a normalized IL-8 level of 0.76 (=[1.08+0.46]/2). Importantly, the therapeutic efficacy of Bortezomib is independent of cell culture or exposure conditions, if the cell-based (number of moles Bortezomib per number of cells) instead of molar dose is considered.

Identical Bortezomib efficacy for ALI and submerged conditions provides validation for ALICE-CLOUD: For ALI exposures, the cell-delivered (or cell-based) dose is a more adequate dose metric than molar dose, since all of the delivered substance is in immediate and direct contact with the cells (13 µm thin liquid layer on cells). Hence, we calculated the cell-based Bortezomib dose, i.e. the amount of drug "seen" by a single cell, for ALI and submerged culture conditions. When we plotted the cell-based Bortezomib dose against the decrease in IL-8 promoter activation(Figure 1, right), we observed very similar sigmoidal dose-response behaviour where 50% of the maximum therapeutic efficacy (TE_{50}) was reached at a dose of 66 pmol Bortezomib per million cells independent of exposure type(aerosol or bulk liquid) and cell culture conditions (ALI and submerged) . These data

clearly show that aerosolized delivery of Bortezomib with the ALICE-CLOUD yields no statistically significant difference in anti-inflammatory therapeutic efficacy compared to liquid application of the drug. Of note, this also confirms that A549 cells are non-polarizing cells showing identical response under ALI and submerged cell culture conditions. Hence, A549 cells are ideal for the ALICE-CLOUD validation study; but they are not recommended for inhalative drug screening studies.

Conclusions

This study shows the potential of the ALICE-CLOUD technology for *in vitro* efficacy and safety testing with novel drugs for inhalation therapy. For the recommended 200 µL of liquid nebulized with the ALICE-CLOUD, the exposure procedure itself had no adverse effect on the viability of the human alveolar epithelial cell line (A549). Dose delivery was repeatable (9.3%), spatially uniform (<4.3% insert-insert variability) and occurred at an aerosol-to-cell delivery efficiency of 16.8% (for 6-well inserts). Combined with an exposure time of 3.5 min, this results in a relatively high drug-to-cell delivery rate of 0.4 µL/cm^2/min.

In a proof-of-concept study, we demonstrated the value of the ALICE-CLOUD for functional analysis of a novel aerosolized drug. As an example for repurposing of drugs, we showed that the FDA-approved proteasome inhibitor Bortezomib (Velcade®) can be aerosolized to efficiently block proteasomal activity and mediate potent anti-inflammatory effects in TNFα-stimulated A549 cells at ALI conditions. There was no statistically significant difference in therapeutic efficacy when compared to the conventional non-aerosolized, submerged cell assay. This validates the ALICE-CLOUD as a useful preclinical tool for dosimetrically accurate and highly reproducible in vitro determination of the delivery efficiency of aerosolized drugs.

The ALICE-CLOUD is a closed system, which can hold one standard multi-well cell culture plate. All wetted parts can be easily sterilized and the entire system can be placed under a clean bench for sterile handling of the samples. The device is an automation-friendly, one-button system, which can be operated reliably without expert knowledge in aerosol technology. Although the ALICE-CLOUD was used here for a pulmonary epithelial cell line, it is also applicable to co-culture models or tissue sections of the lung or any other epithelial barrier (e.g. skin).

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