Research Article

Safety Assessment of Excipients (*SAFE*) for Orally Inhaled Drug Products

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Abstract

The development of new orally inhaled drug products requires their demonstration of safety, which must be proven in animal experiments. New *in vitro* methods may replace, or at least reduce, these animal experiments, provided they are able to correctly predict safety or possible toxicity in humans. However, the challenge is to link *in vitro* data obtained in human cells to human *in vivo* data. We here present a new approach to the safety assessment of excipients (*SAFE*) for pulmonary drug delivery. The *SAFE* model is based on a dose response curve of 23 excipients tested on the human pulmonary epithelial cell lines A549 and Calu-3. The resulting *in vitro* IC50 values were correlated with the FDA-approved concentrations in pharmaceutical products for either pulmonary (if available) or parenteral administration. Setting a threshold of 0.1% (1 mg/mL) for either value yielded four safety classes and allowed to link IC50 data as measured in human cell cultures *in vitro* with the concentrations of the same compounds in FDA-approved drug products. The necessary *in vitro* data for novel excipients can be easily generated, and the *SAFE* approach allows putting them into context for eventual use in human pulmonary drug products. Excipients that are most likely not safe for use in humans can be excluded early on from further pharmaceutical development. The *SAFE* approach thus helps to avoid unnecessary animal experiments.

1 Introduction

The enormous expense of and the ethical need to reduce animal experiments during preclinical trials has led to the implementation of *in vitro* tests in European Medicines Agency (EMA) and Organization for Economic Co-operation and Development (OECD) guidelines (EMA, 2016; OECD, 2000), and the Food and Drug Agency (FDA) Guidance for Industries "Drug Metabolism/Drug Interaction Studies in the Drug Development Process: Studies *In Vitro*" recommends *in vitro* studies of the safety and efficacy testing of potential drugs to exclude any toxic or non-effective drugs at an early stage and thus reduce the risk of failing during clinical trials (FDA, 1997). However, although regulatory agencies encourage the use of *in vitro* assays for the screening and evaluation of new drug formulations, animal testing is still the standard procedure to evaluate inhaled drug products (Silva and Sørli, 2018). Mice, rats, dogs and non-human primates are the most widely used animals for this purpose (Pritchard et al., 2003).

The total costs of drug development have increased from $800m to $2,000m per drug, whereby drug development for delivery via inhalation has reached average costs of $1,134m per new drug formulation (Adams and Van Brantner, 2006). In 2004, the FDA founded the Critical Path Initiative, which is a project aimed at optimizing the costly drug development process. In the process, the determination of the safety and efficacy of new drug formulations was found to be a main cost contributor (Woodcock and Woosley, 2008). Indeed, the costs of animal testing during drug development lie between $430m and $1,098m per drug (DiMasi et al., 2016).

Next to the financial and ethical aspects, the questionable prediction of the human response by data from animal experiments is an ongoing discussion (Bracken, 2009; Fröhlich, 2017). Safety issues account for 24% of clinical study terminations (Harrison, 2016). According to Li (2004), some reasons for this uncertainty might be that animals have different toxic and detoxifying molecular mechanisms and thus have a different sensitivity to com-

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pounds compared with humans. In addition, studies are limited in the investigation of toxic endpoints, the number of tested individuals is restricted, and studies struggle with dose adaptation.

An example of a successfully established *in vitro* assay is the skin sensitization assay based on the stimulation of different human cell lines (OECD, 2018a). However, while there has been progress in the development of some *in vitro* assays, there is still a lack of adequate cell- and tissue-based *in vitro* models for other organs such as the respiratory tract. An OECD guideline (OECD, 2018b) now defines the certification process and the required quality controls for *in vitro* tests that serve the future evaluation of the assessment of human safety.

To face the challenge of developing an adequate *in vitro* model of the respiratory tract for safety evaluation, especially to assess nanoparticle (NP) deposition in the lung, the development of complex cell culture systems using two or more cell lines is gaining ground (Chary et al., 2018). But, no systematic data sets for the toxicological assessment of nanoparticles are available due to non-standardized parameters, different testing systems (cells, animals), and the different types and characteristics of the particles themselves (Mahmoudi et al., 2012). The many different endpoints of *in vitro* data obtained during safety studies – cell toxicity, proinflammatory reactions, translocation of nanoparticles in the tissue, and the resulting uptake mechanism, to name a few – should be evaluated in a comprehensive way to enable their inclusion into a guideline process (Drasler et al., 2017).

The correlation of the *in vitro* and *in vivo* activity of nanoparticles (NP), such as TiO_2, gold and polystyrene particles, was investigated by Rushton et al. (2010). Their *in vitro* assay, which was based on the secretion of reactive oxygen species (ROS), correlated significantly with *in vivo* observations (PMN recruitment) with an R^2 of 0.81. They proposed an NP hazard scale based on the *in vivo* activity of the NP surface area (Rushton et al., 2010). However, such a hazard classification related to risk (or safety) assessment of NP is still not fully established due to a lack of data on exposure processes, biokinetics, and organ-specific lung toxicity (Oberdörster, 2010; Upadhyay and Palmberg, 2018).

While the elucidation of adverse outcome pathways (AOPs) with their related cellular, tissue/organ and organism/population level key events might help to predict the toxicity of inhaled substances (Clippinger et al., 2018a; Halappanavar et al., 2019), there is still no safety classification system of substances used for oral inhalation products, including excipients, i.e. bulking agents, that are already approved by FDA for some marketed drug products. However, even larger datasets, such as those obtained by some "omics"-technologies, so far cannot overcome all limitations of *in vitro* and *in vivo* generated data. Therefore, especially the prediction of adverse effects remains a challenge (Ghallab and Bolt, 2014).

An important first step towards such an *in vitro* based safety classification might be the comparison of *in vitro* data with LD50 values, which are used as a basis for hazard classification (Strickland et al., 2018). In particular, the *in vitro* MTT assay has shown high sensitivity with regard to the cytotoxic effects of substances, generating a simple and reproducible determination of an IC50 value (Scherließ, 2011). Sauer et al. (2013) correlated *in vivo* LD50 and *in vitro* IC50 toxicity data from standardized MTT assays for 19 chemicals in order to categorize them into four hazard classes that predict their acute inhalative toxicity *in vivo* on the basis of the *in vitro* data. The resulting comparison allowed the identification of harmful substances. However, this classification system relates *in vitro* data to animal *in vivo* data and not to human clinical data.

In order to bridge the gap between human *in vitro* and human clinical data, we attempted to correlate these directly. The *in vitro* cytotoxicity of 23 excipients, which may be employed in an FDA-approved concentration range (i.e. considered as safe), was assessed and used to set up a four-quadrant analysis to classify the safety of other excipients to be potentially used in orally inhaled drug products.

2 Material and methods

2.1 Cell lines

The human cell line A549 (ACC107; Lieber et al., 1976) was obtained from DSMZ and was cultivated in RPMI 1640 (Roswell Park Memorial Institute 1640, gibco™, Fisher Scientific, USA) supplemented with 10% FBS (fetal bovine serum, South American origin, PAN-Biotech, Germany) and 1% antibiotics (penicillin (10,000 U/mL) / streptomycin (10,000 µg/mL), gibco™, Fisher Scientific, USA). The human cell line Calu-3 (ATCC® HTB 55™; Fogh et al., 1977) was cultured in MEM (minimal essential medium, gibco™, Fisher Scientific, USA) supplemented with 10% FBS, 1% 100x MEM NEAA (non-essential amino acids solution, gibco™, Fisher Scientific, USA), 1% 100 mM sodium pyruvate solution (gibco™, Fisher Scientific, USA) and 1% antibiotics. The A549 and Calu-3 cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C to passage number 50.

2.2 Selection of the test substances

The 23 substances listed in Table 1 together with the corresponding supplier and their solubility in water were used in the study. All substances are approved for a specified concentration range in parenteral or pulmonary drug application according to the Inactive Ingredient Search for Approved Drug Products[1]. A compilation of the approved concentrations from this database is given in Table 2. A pulmonary-approved concentration was available for citric acid monohydrate, citric acid anhydrous, glycerol, L-ascorbic acid, polysorbate 80, and sodium chloride. Where no data for inhalation were given, the parenteral concentration was used.

2.3 Determination of the *in vitro* IC50 value

Preparation of test concentrations

Standardized MTT assays were performed to determine the *in vitro* IC50 using several dilutions of the test compounds. In the first round of assays, the concentration range was narrowed to

[1] https://www.accessdata.fda.gov/scripts/cder/iig/index.cfm

Tab 1: List of test substances with the related CAS number, supplier and solubility in water

Substance	CAS number	Supplier	Water solubility
Albumin from human serum	70024-90-7	Baxalta, Shire, Germany	200 g/L (20°C)
Benzoic acid	65-85-0	Roth, Germany	2.9 g/L (20°C)
Benzyl alcohol	100-51-6	Fisher Scientific, United Kingdom	40 g/L (20°C)
Citric acid monohydrate	5949-29-1	Roth, Germany	50 g/L (20°C)
Citric acid anhydrous	77-92-9	PanReac AppliChem ITW Reagents, Germany	100 g/L (20°C)
Docusate sodium	577-11-7	Sigma Aldrich, USA	15 g/L (25°C)
Glycerol	56-81-5	PanReac AppliChem ITW Reagents, Germany	Fully miscible
L(+)-Ascorbic acid	50-81-7	VWR Chemicals Belgium	50 g/L (20°C)
L-Alanine	56-41-7	PanReac AppliChem ITW Reagents, Germany	100 g/L (20°C)
L-Arginine	74-79-3	Sigma Aldrich, USA	150 g/L (20°C)
L-Cysteine	52-90-4	Roth, Germany	25 g/L (20°C)
L-Methionine	63-68-3	Sigma, Germany	25 g/L (20°C)
L-Proline	147-85-3	Roth, Germany	1500 g/L (20°C)
Palmitic acid	57-10-3	Merck, Germany	insoluble
Poloxamer 188 (Kolliphor® 188)	/	Sigma Aldrich, USA	No data available
Polyethylene glycol 200 (PEG 200)	25322-68-3	Merck, Germany	70 g/L (20°C)
Polyethylene glycol 300 (PEG 300)		Super refined™, CRODA, United Kingdom	soluble
Polyethylene glycol 400 (PEG 400)		Rotipuran®, Roth, Germany	No data available
Polyethylene glycol 600 (PEG 600)		Super refined™, CRODA, United Kingdom	soluble
Polysorbate 80 (Tween-80)	9005-65-6	Sigma Aldrich, Switzerland	Fully miscible
Polysorbate 80 (HX2)		NOF CORPORATE, Japan	No data available
Polysorbate 20 (Tween 20)	9005-64-5	Super refined™, CRODA, United Kingdom	Fully miscible
Sodium chloride	7647-14-5	Roth, Germany	> 300 g/L (20°C)

approximate the IC50. The initial concentrations were chosen based on the *in vitro* classification scheme (Sauer et al., 2013) of different cell culture systems, starting from 0.1 and continuing up to 10 mg/mL. Dilutions were prepared in HBSS (Hank's Balanced Salt Solution, gibco™, Fisher Scientific, USA) the day before the MTT assay and stored at 4°C. In order to specify the IC50 value in further experiments, a total of three cycles of MTT assays were performed for both cell lines. Where the IC50 of an excipient exceeded 10 mg/mL, the substance was employed up to its maximal solubility in water (Tab. 1). For the relative representation of the *in vitro* IC50, 1 g (undiluted substance) was set as the 100% mark to enable unit equivalence to the FDA concentration.

Cell viability measurements

$2x10^5$ cells/mL A549 or Calu-3 were seeded in a 96-well plate (Greiner Bio-one, Germany) in 200 µL medium. 24 h later, the cells were visualized by light microscopy (PrimoVert, Zeiss, Germany) to verify epithelial confluence of nearly 100%. The cells were washed twice with HBSS and 200 µL of the test substances were applied. After incubation for 4 h on a shaker at 35 rpm and 37°C, the cells were washed once with HBSS. 0.5 mg/mL MTT reagent (methylthiazolyldiphenyl-tetrazolium bromide, Acros Organics, USA) was added for 4 h at 37°C and 35 rpm and protected from light. Absorbance was measured at 550 nm with a plate reader (Synergy 2, BioTek Instruments GmbH). To calculate the cell viability after substance exposure, a positive control of 1% Triton X-100 (PanReac AppliChem ITW Reagents, Germany) and a negative control of HBSS were used as described in Formula 1.

Formula 1: Calculation of the cell viability based on the absorbance measurements obtained from the MTT assay

$$Viability\ [\%] = \frac{(absorbance_{test\ substance} - absorbance_{1\%\ Triton\ X-100})}{(absorbance_{HBSS} - absorbance_{1\%\ Triton\ X-100})} \times 100$$

Tab. 2: List of excipients with their associated parenteral and pulmonary FDA-approved concentration from the Inactive Ingredient Search for Approved Drug Products
"No approved concentration" means that no FDA-approved product was identified.

Substance	CAS number	FDA-approved concentration range – parenteral	FDA-approved concentration range – pulmonary
Albumin from human serum	70024-90-7 FDA:9048468	0.1-2% 80% (powder for injection solution, lyophilised)	no approved concentration
Benzoic acid	65-85-0	0.0031-5%	no approved concentration
Benzyl alcohol	100-51-6	0.4-18% 9.45 mg/mL Powder for injection: 10.4 mg/mL	no approved concentration
Citric acid monohydrate	5949-29-1	0.05-38.46% 2.2-5.2 mg/mL Powder for injection: 384.46 mg	0.028% 4.04 mg/Inh 4.2 mg/mL 0.002-4.04 mg/Inh
Citric acid anhydrous	77-92-9	1-7% Powder for injection: 42.19% 2-10 mg/mL	0.56 mg/2 mL 0.0003-0.027%
Docusate sodium	577-11-7	intramuscular: 0.015%W/V	no approved concentration
Glycerol	56-81-5	2.5-15.36% 18.82 mg/mL	7.3%
L(+)-Ascorbic acid	50-81-7	50.4-62.5% Powder for injection: 0.088-48%	959.5 mg/Inh 0.11-1.02%
L-Alanine	56-41-7	no approved concentration	no approved concentration
L-Arginine	74-79-3	5-39% Powder for injection: 14-78%, 70.7 g	no approved concentration
L-Cysteine	52-90-4	0.01-0.1% Powder for injection: 2.6%	no approved concentration
L-Methionine	63-68-3	0.004-49.2%	no approved concentration
L-Proline	147-85-3	0.34-35.6%	no approved concentration
Palmitic acid	57-10-3	0.001%	no approved concentration
Poloxamer 188 (Kolliphor® 188)	/	0.2-0.6%	no approved concentration
Polyethylene glycol 200 (PEG 200)	25322-68-3	intramuscular: 30%	no approved concentration
Polyethylene glycol 300 (PEG 300)		4.42-65% 320 mg/5 mL 650 mg/1 mL	no approved concentration
Polyethylene glycol 400 (PEG 400)		0.49-75.58% 0.67 mL/1 mL	no approved concentration
Polyethylene glycol 600 (PEG 600)		5%	no approved concentration
Polysorbate 80 (Tween-80)	9005-65-6	0.5-63% 260 mg/1 mL 400 mg/ 5mL	0.02 μg 0.22 mg 0.37 mg/2 mL 0.02-0.04%
Polysorbate 80 (HX2)			
Polysorbate 20 (Tween 20)	9005-64-5	0.003-4.8% Powder for injection: 0.044% 10 mg/mL	no approved concentration
Sodium chloride	7647-14-5	0.9-90% 9-18 mg/1 mL 801.1 μl/1 mL	11.25 mg/5 mL 8 mg/1 mL 8.5-27 mg/3 mL 0.9-1.13%

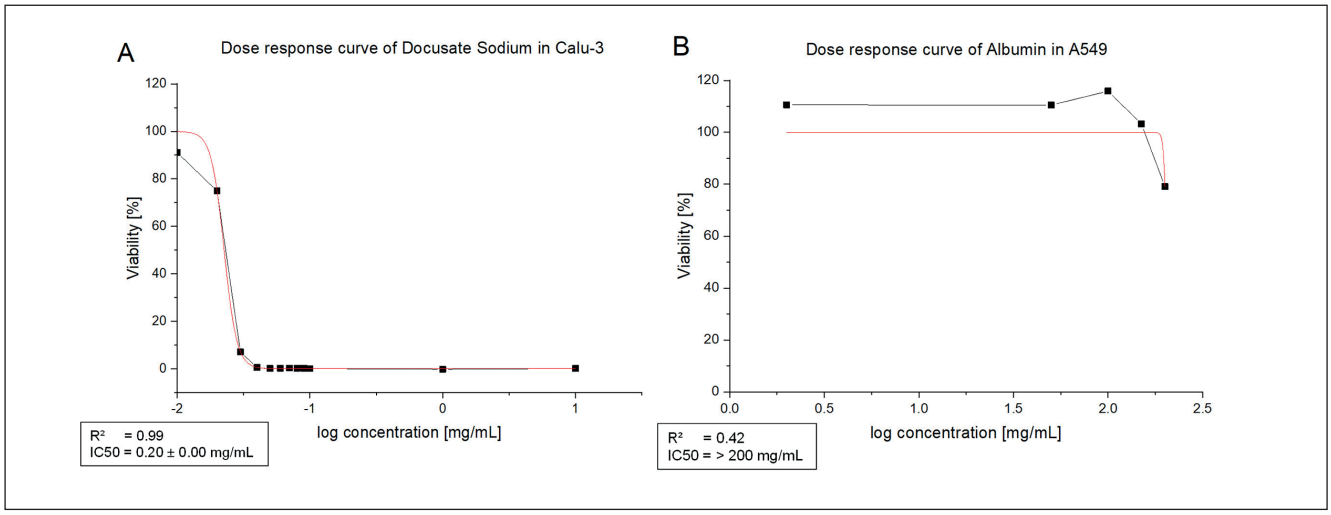

Fig. 1: Exemplary presentation of the dose response curve of docusate sodium in Calu-3 and albumin from human serum in A549 for determination of the IC50 *in vitro*

The applied concentration is given in mg/mL, whereby the maximal concentration is set to 1 g (undiluted substance). The red line indicates the sigmoidal fit used for calculation of the IC50 by OriginPro® 2019. (A) Dose response curve of docusate sodium tested on Calu-3 cells; the IC50 is 0.02%. The coefficient of determination (R^2) is 0.99, indicating a good fit. (B) Dose response curve of albumin tested on A549 cells; the IC50 cannot be determined even up to the maximal solubility of 200 mg/mL.

Generation of dose response curves and calculation of the in vitro *IC50*

The software OriginPro® 2019 (additive, Germany) was used for IC50 calculations based on a dose response curve. The concentrations were applied in a logarithmic scale. A sigmoidal fit was performed with a top asymptote set to 100% viability and the bottom asymptote to 0% viability.

2.4 Statistics

For the IC50 determination performed on Calu-3 cells, 3 technical replicates (wells) in 3 independent experiments for each investigated concentration were performed. The IC50 values calculated from A549 data were based on n = 3 wells per concentration in several experiments performed to determine the IC50 and narrow the concentrations tested next to it. Details are listed in the supplementary information in each graph. Data are summarized as the mean ±SD. Dose response curves and the correlation analysis were performed with the software OriginPro® 2019. A linear fit without weighting parameters was performed to calculate the Pearson r correlation coefficient.

3 Results

3.1 IC50 of excipients

The effects of the excipients on the viability of Calu-3 and A549 were determined for 23 excipients with a standardized MTT assay. The IC50 is given in % and scaled logarithmically. Two examples of dose response curves and the calculation of the tested excipient are shown for docusate sodium (CAS: 577-11-7) on Calu-3 cells (Fig. 1A) and albumin from human serum for A549 (Fig. 1B). Docusate sodium has an IC50 of 0.02% tested on Calu-3 cells. For albumin, no determination of the IC50 value on A549 was possible (IC50 > 200 mg/mL). The dose response curves with the IC50 calculation for all tested excipients are shown in Figures S1 and S2[2].

The *in vitro* IC50 values are listed in Table 3. For the substances albumin from human serum, L-alanine, L-cysteine, L-methionine, palmitic acid, poloxamer 188, and the polyethylene glycols (PEG) 200-600, no calculation of the IC50 was possible in their aqueous solubility range.

No converging fit was obtained in the testing of glycerol (Calu-3), L-proline (A549), and sodium chloride (A549 and Calu-3), but an approximation of the IC50 by the software was possible. *In vitro* calculation of the IC50 was successful for the remaining 11 excipients. The results were compared to the classification by Sauer et al. (2013) (Tab. 3). A comparison of A549 and Calu-3 showed that the observed IC50 values were in a similar range but did not correlate in a regression analysis (Fig. S3[1]; Tab. S2[1]).

3.2 Linear correlation of FDA-approved concentration range and *in vitro* IC50

For evaluation of the linear correlation between the *in vitro* IC50 value and the approved FDA concentration, regression analysis was performed.

Calu-3 IC50 values (Tab. 3) of the substances benzyl alcohol, citric acid monohydrate, citric acid anhydrous, docusate sodium, glycerol, L-ascorbic acid, L-arginine, L-cysteine, L-proline,

[2] doi:10.14573/altex.1910231s

Tab. 3: List of excipients with the IC50 [%] in A549 and Calu-3 cells in comparison to the classification by Sauer et al. (2013)

Substance	CAS number	A549 IC50 *in vitro* [%]	Calu-3 IC50 *in vitro* [%]	Classification according to Sauer et al. (2013)
Albumin from human serum	/	> 20 (solubility reached)	> 20 (solubility reached)	Class 4
Benzoic acid	65-85-0	0.048 ±0.100	not measured	Class 2
Benzyl alcohol	100-51-6	0.344 ±0.013	0.305 ±0.032	Class 3
Citric acid monohydrate	5949-29-1	0.095 ±0.003	0.038 ±0.001	Class 2
Citric acid anhydrous	77-92-9	0.018 ±0.003	0.032 ±0.001	Class 2
Docusate sodium	577-11-7	0.020 ±0.001	0.002 ±0.000	Class 1-2
Glycerol	56-81-5	38.875 ±0.054	Fit does not converge (range 1-25)	Class 4
L(+)-Ascorbic acid	50-81-7	0.092 ±0.002	0.017 ±0.003	Class 2
L-Alanine	56-41-7	> 10	> 10	Class 4
L-Arginine	74-79-3	2.162 ±0.220	0.946 ±0.070	Class 4
L-Cysteine	52-90-4	> 2.5	1.214 ±0.087	Class 4
L-Methionine	63-68-3	> 2.5	> 2.5	Class 4
L-Proline	147-85-3	Fit does not converge (range 19-25)	11.651 ±0.629	Class 4
Poloxamer 188 (Kolliphor® 188)	/	solubility reached	solubility reached	Class 4
Polyethylene glycol 200 (PEG 200)	25322-68-3	> 10	> 10	Class 4
Polyethylene glycol 300 (PEG 300)				Class 4
Polyethylene glycol 400 (PEG 400)				Class 4
Polyethylene glycol 600 (PEG 600)				Class 4
Polysorbate 80 (Tween-80)	9005-65-6	0.147 ±0.017	1.117±0.071	Class 3
Polysorbate 80 (HX2)		0.118 ±0.007	0.224 ±0.030	Class 3-4
Polysorbate 20 (Tween 20)	9005-64-5	0.024 ±0.009	0.120 ±0.011	Class 2-3
Sodium chloride	7647-14-5	Fit does not converge (range 1-10)	Fit does not converge (range 1-10)	Class 4

polysorbate 80, polysorbate 80 HX2, polysorbate 20, and sodium chloride were plotted against the approved FDA concentration (Tab. 2). The percentage ranges of the FDA concentrations given for the pulmonary and parenteral application were included. Figure 2 shows a positive slope of the regression line of 0.42 ±0.12 and a coefficient of determination (COD, R^2) of 0.31, indicating a poor correlation.

Next, a regression analysis was performed for the A549 cell line (Fig. 3). The IC50 values (Tab. 3) and the FDA-approved concentrations (Tab. 2) for benzoic acid, benzyl alcohol, citric acid monohydrate, citric acid anhydrous, docusate sodium, glycerol, L-ascorbic acid, L-arginine, L-proline, polysorbate 80, polysorbate 80 HX2, polysorbate 20, and sodium chloride were plotted. A positive slope of the regression line of 0.60 ±0.12 was obtained by performing a correlation analysis with an R^2 of 0.49. In comparison to the correlation using the Calu-3 cell line, the R^2 indicates in the A549 plot a slightly better correlation.

3.3 The safety assessment for excipients (SAFE)

3.3.1 Safety assessment based on the IC50 *in vitro* and the FDA-approved concentration

The drawback of the linear regression analysis using the FDA data is that there might be some buffer for a higher approved concentration as the approved value cannot be compared with an LD50 derived from animals. However, knowledge from the Sauer et al. (2013) publication can be utilized to consider known hazard effects. Using the classification suggested in that paper on the IC50 values of the tested excipients against the FDA-approved concentration range, a four-class division was obtained.

The concentration of 0.1% (1 mg/mL) was determined as a critical concentration for the IC50 due to the first occurrence of toxicological effects in the cellular test system according to the acute toxicity classification of the UN (class #1-2: fatal if inhaled, class #3: toxic if inhaled, class #4-5: harmful and maybe

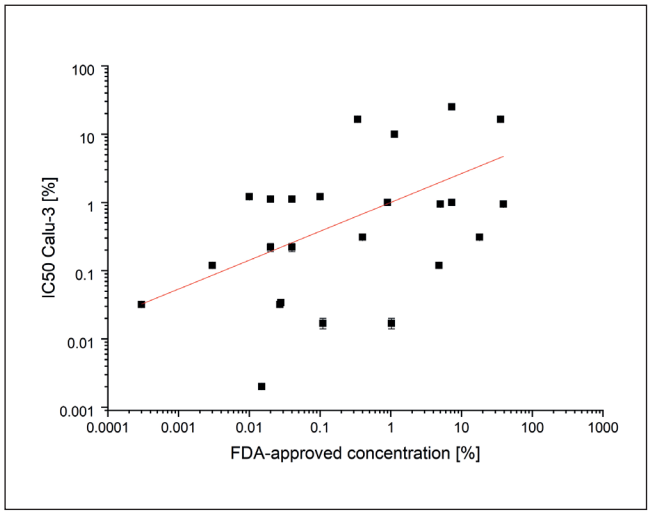

Fig. 2: Regression analysis of IC50 [%] *in vitro* in Calu-3 cells vs FDA approved concentration range [%]

The concentration of the calculated *in vitro* IC50 values was based on a maximal concentration of 1 g. The approved concentrations were obtained from the FDA's database on inactive ingredients. The values for the linear regression analysis are: slope: 0.42 ±0.13, sum square of errors: 20.90; Pearson R: 0.55; coefficient of determination (COD, R^2): 0.31; Correlation R^2: 0.28.

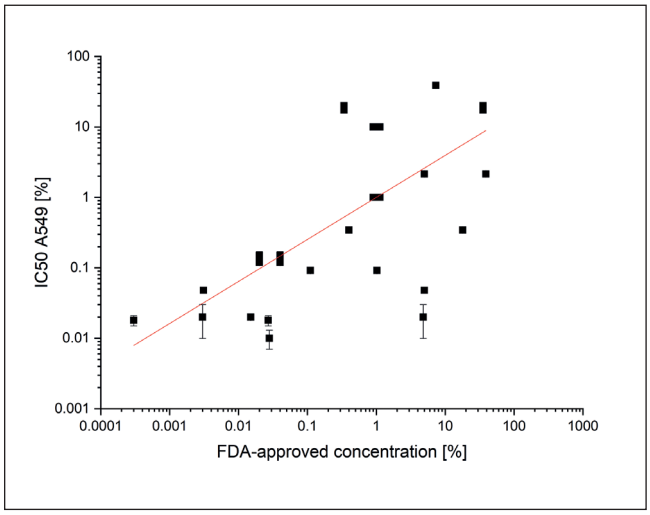

Fig. 3: Regression analysis of IC50 [%] *in vitro* in A549 cells vs approved FDA concentration range [%]

The concentration of the calculated *in vitro* IC50 values was based on a maximal concentration of 1 g. The approved concentrations were obtained from the FDA's database on inactive ingredients. The values of the linear regression analysis are: slope: 0.60 ±0.12, sum square of errors: 18.24; Pearson R: 0.70; coefficient of determination (COD, R^2): 0.49; Correlation R^2: 0.47.

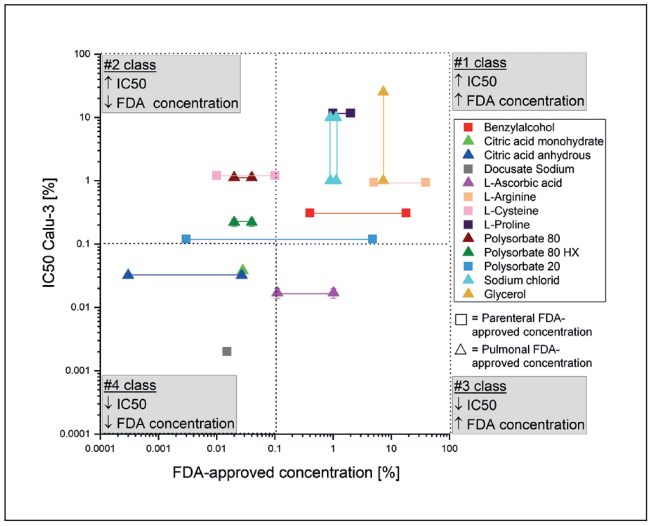

Fig. 4: The IC50 values of the excipients tested in the Calu-3 cell line and the FDA-approved concentration ranges can be set up in a four-class system

The diagram consists of the calculated IC50 values plotted against the approved FDA concentrations (squares: parenteral approved concentration, triangles: pulmonary approved concentration) for the tested excipients. Dividing this diagram into four regular squares from 0.1% IC50 to 0.1% FDA and up to 100% IC50/FDA, results in a four-class system. #1 class: high IC50, high FDA-approved concentration, #2 high IC50, low FDA-approved concentration, #3 class: low IC50, high FDA-approved concentration, #4 class: low IC50, low FDA-approved concentration.

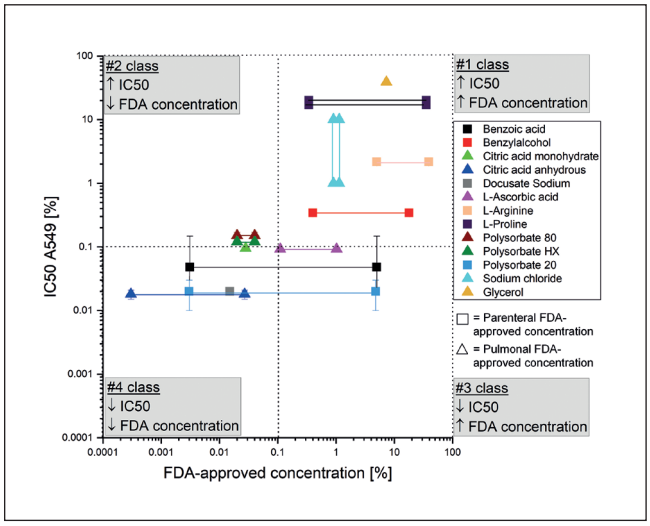

Fig. 5: The IC50 values of the excipients tested in the A549 cell line and the FDA-approved concentration ranges can be set up in a four-class system

The diagram consists of the calculated IC50 values plotted against the approved FDA concentrations (squares: parenteral approved concentration, triangles: pulmonary approved concentration) for the tested excipients. Dividing this diagram into four regular squares from 0.1% IC50 to 0.1% FDA and up to 100% IC50/FDA, results in a four-class system. #1 class: high IC50, high FDA-approved concentration, #2 high IC50, low FDA-approved concentration, #3 class: low IC50, high FDA-approved concentration, #4 class: low IC50, low FDA-approved concentration.

Tab. 4: Line-up of the four class-based classification by Sauer et al. (2013) and the tested concentrations ranges for the SAFE-approach in %

In vitro hazard class	Concentration range for cell monolayer [mg/mL] (Sauer et al., 2013)	Concentration range for *SAFE*-classification [%]
# 1	< 0.1	< 0.01
# 2	0.1-1	0.01-0.1
# 3	1-10	0.1-1
# 4	> 10	1

harmful if inhaled (United Nations, 2017)). The excipients with an IC50 below 0.1% were categorized in classes 1 and 2 according to Sauer et al. (2013) (Tab. 4). Above an IC50 of 0.1%, no safety concerns are obvious, so that a resulting classification into class 4 is likely. The corresponding classification of all tested excipients according to the system proposed by Sauer et al. (2013) is shown in Table 3.

A four-class system is obtained by setting 0.1% as the limit line of cellular toxicity effects and outlining evenly distributed squares in the fit of IC50 and the approved FDA concentration. In class 1, the *in vitro* IC50 and the FDA-approved concentration are consistently high. For the Calu-3 cell line, benzyl alcohol, glycerol, L-proline, sodium chloride, L-arginine and, in the border region, polysorbate- 20, were categorized as class 1. These excipients are non-toxic according to the system of Sauer et al. (2013). In the A549 cell line, class 1 substances have similar values, except polysorbate 20, which is categorized as class 3-4. Class 2 is the quadrant to the left, which is characterized by a high IC50 value and a lower FDA-approved concentration. Polysorbate 80, polysorbate 80 HX2, polysorbate 20, and L-cysteine tested on Calu-3 cells, and polysorbate 80 and polysorbate 80 HX2 tested on A549 cells fall into class 2. Class 3 is characterized by a high FDA-approved concentration and a low IC50 value. L-ascorbic acid tested on Calu-3 belongs to this class, whereas in A549 L-ascorbic acid, benzoic acid and polysorbate 20 are classified here. Class 4 contains the squares at the intersection with a low IC50 value and a corresponding low FDA concentration. Citric acid, docusate sodium, and benzoic acid fall into this category for Calu-3, with an additional test of benzoic acid and citric acid on the limital zone to class 3 on A549. This classification system is visualized for Calu-3 in Figure 4 and for A549 in Figure 5.

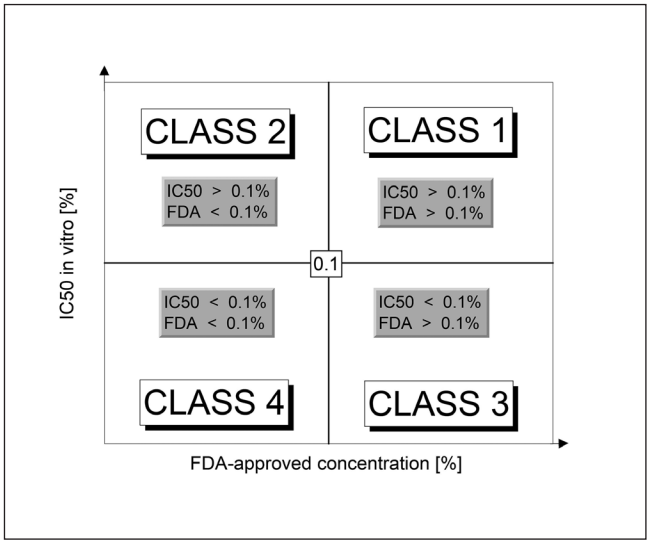

Fig. 6: The *SAFE* classification
Divided into 4 classes, *SAFE* gives an indication of the categorization of the *in vitro* IC50 value to the FDA-approved concentration. Arrangement of the 4 classes: Class 1: IC50 is higher than 0.1%, the correlated FDA concentration is higher than 0.1%; Class 2: IC50 is higher than 0.1%, the correlated FDA concentration is lower than 0.1%; Class 3: IC50 is lower than 0.1%, the correlated FDA concentration is higher than 0.1%; Class 4: IC50 is lower than 0.1%, the correlated FDA concentration is lower than 0.1%.

3.3.2 Consequences of the four classes for safety assessment of excipients (SAFE)

The *SAFE* classification can help to estimate, based on *in vitro* IC50 data, whether pulmonary administration to humans will be safe or not. This estimation is based on the results presented in Figures 4 and 5. The concentration of 0.1% (which indicates problems with the animal toxicity study in the preclinical phase) forms the center of the coordinate system and is the dividing line for the resulting classification (Fig. 6).

A higher IC50, which is above 0.1% (classes 1 and 2), is worth considering for further development. A lower IC50 below 0.1%, which is here referred to as classes 3 and 4, is not recommended for further development. The target class 1 implements a high IC50 and a high FDA-approved concentration, making it the most appropriate class to continue with further testing and potential use in humans. Most tested excipients are classified in this non-toxic category and no safety issues are expected during subsequent pre-clinical and clinical development. In class 2, the tested *in vitro* concentration could be increased in view of a higher FDA-approved concentration. In classes 3 and 4, in correspondence with the classification by Sauer et al. (2013), cytotoxicity is obvious, and therefore considering such compounds for further development steps may be risky. For a class 4 substance, the approval requirements are likely to be complicated.

The *SAFE* system helps to estimate the risk of excipients and the chance of obtaining FDA approval by using them in new drug formulations.

4 Discussion

The concept of an *in vitro*-*in vivo*-correlation (IVIVC) aims to predict the bioavailability and the efficacy of the tested drug product based on its release kinetics (Barakat et al., 2015; Shen and Burgess, 2016) in relation to the Biopharmaceutical Classification System (BCS) adapted by the FDA (CDER/FDA, 2017). In line with this classic BCS, a pulmonary biopharmaceutical classification (pBCS) was described, implementing the impacts of lung biology in terms of lung metabolism, drug-drug interactions, presence of transporters and mucus, protein binding, clearance, surfactant, and formulation properties like size, solubility, and used excipients (Hastedt et al., 2016; Gonda, 2006). Despite the consideration of all these physiological aspects, no linear IVIVC can predict the *in vivo* response in the lung on the basis of an *in vitro* dose dependency (Eixarch et al., 2010). Therefore, an *in vitro* testing strategy for predicting the respiratory toxicity of chemicals and drug products is still needed. One initial step toward this goal is the development of a classification system to predict the safety of chemicals and drug products.

The present work is an attempt to break down the complexity of the physiological aspects of the lung to gradually approach an *in vitro* test strategy for orally inhaled drug products that can support formulation development by addressing cytotoxicity towards pulmonary epithelial cells. Sauer et al. (2013) already established an *in vitro* hazard classification system by correlating IC50 values of 19 substances tested in 3 cellular systems for different incubation periods (A549 – 24 hours; EpiAirway™ – 3 hours; MucilAir™ system – 24 hours) with the GHS classification. We slightly modified the experimental setup of Sauer et al. (2013) by shortening the incubation period to 4 h, in order to consider the exposure period mentioned in the OECD acute inhalation toxicity guideline 436 (OECD, 2009), and by using A549 and Calu-3 cells. Challenge of the cells with the test compounds in a salt buffer for 4 instead of 24 h was intended to reduce the influence of both cell proliferation and the incubation medium on the results.

The resulting *in vitro* hazard classification includes different concentration ranges for the cellular systems to predict GHS respiratory category. Independent of the chosen cell system and incubation period, a concentration of 0.1% was in all cases associated with GHS category ≤ 3. The classification according to Sauer et al. (2013) formed the basis for the *in vitro* IC50 limiting value of 0.1% of the *SAFE* classification. In summary, there is no guarantee to have a safe compound when the IC50 is above the threshold of 0.1%, but it is a clear indication. We recommend when developing a new drug product also to test prolonged incubation periods and other cell types as applicable.

To obtain an IVIVC based on the *in vitro* IC50 evaluation, we attempted to correlate the *in vitro* data on the excipients with the *in vivo* LD50 based GHS classification. A direct comparison with the GHS classification based on oral LD50 data is not possible for pharmaceutical excipients as most of them are classified as safe (GHS class 4/5, see Tab. S1[1]). However, upon compilation of the GHS classification for acute pulmonary toxicity of the tested excipients (Tab. S1[1]), we found a large data gap for pulmonary LD50 values, so that no effective, direct correlation of the IC50 values with the *in vivo* data on pulmonary toxicity was possible (ECHA, search for chemicals, guidance on the safe use of the substance; Rowe et al., 2009; GESTIS *Stoffdatenbank*; search in NICEATM Integrated Chemical Environment data base (ICE)).

For pulmonary classification, the exposure route is divided into gases, vapors, dusts and mists (United Nations, 2017). No increased GHS classes were obtained for these application forms. Gases of benzyl alcohol were assigned GHS Class 1, while docusate sodium, sodium chloride, and polysorbate 20 were assigned to GHS Class 2. This use of different routes of administration *in vivo* makes it difficult to achieve a standardized *in vitro* comparison.

More complex cell culture systems have been developed with the aim of achieving a better IVIVC (Fizeşan et al., 2018). By testing the cytotoxicity of inhaled drug products in a commercially available human 3D cellular model of the lung (MucilAir), Sivars et al. (2018) found a good correlation of *in vitro* respiratory toxicity data based on measurement of cell barrier integrity, cell viability, ciliary beating frequency (CBF), mucociliary clearance, and the resulting cytokine release, with *in vivo* toxicity data.

In the mentioned study, MucilAir was cultivated under serum-free conditions to avoid the use of FCS, which is associated with pain and suffering of the animals from which it is obtained. The amount and type of serum proteins is of particular importance as they can influence the binding and activity of compounds and nanomaterials (Drasler et al., 2017; Moore et al., 2015). Therefore, we challenged the cells in salt buffer without FCS, but also intend to replace FCS in the culture medium by defined supplements in future studies.

In addition to the more complex 3D cell culture systems like MucilAir, attempts have been made in recent years to recreate the physiology of the lung using microfluidic systems. Huh et al. (2011) were able to reconstitute the toxic and inflammatory responses of the lung in comparison to exposure data of silica particles from mice by using a "lung-on-a-chip" device. However, more complex systems such as co-cultures or chip-based systems are challenging to validate (Huh et al., 2011; Dipasri et al., 2016).

The *in vitro* test strategy we followed in this study might be extended and combined with further specific methods (e.g., surfactometry) and summarized into a safety assessment of the test compound. Such integrated test strategies for the *in vitro* prediction of acute inhalation toxicity were discussed in 2018 at the workshop "Alternative approaches for acute inhalation toxicity testing to address global regulatory and non-regulatory data requirements" (Clippinger et al., 2018b). The paper summarizes the current state of inhalative *in vitro* technologies as well as the criteria that must still be met to overcome the obstacles for guideline acceptance, such as an information transfer from animal experiments, the correct application of dosimetry and realization to industrial applications with their resulting technical needs.

The toxic effects of substances in the lung depend on different parameters, such as airborne concentration, particle size, solubility in surfactant, reactivity, air exchange, rate of exposure, interactions with other inhaled substances, and the specific immunological response (Bakand et al., 2005). In particular, the consideration of the immunological reaction of the test system is a major part of the safety assessment of a compound. By setting up a tetraculture of A549, HMC-1, THP-1, and EA.hy 926 and using the Vitrocell® CLOUD system, Klein et al. (2013) could show that inflammatory responses are overpredicted under submerged conditions. This could be an explanation for cases where the FDA-approved concentration was high but the IC50 value was low (*SAFE* class 3). To avoid this overestimation, air-liquid interface (ALI) exposure systems – potentially covered with mucus or surfactant – and the dosimetry should be taken into account (Paur et al., 2011).

In order to investigate nanomaterials, existing *in vitro* assays such as the NR8383 alveolar macrophage assay described by Wiemann et al. (2018) can be combined with *SAFE*, taking ongoing discussions on dosimetry (Wiemann et al., 2018; Schmid and Cassee, 2017) and experimental setups (Kong et al., 2011) into account. Dosimetry is an important aspect of focusing the deposition of particles or substances in the alveolar region. Donaldson et al. (2008) obtained a high IVIVC by expressing the dose in terms of A549 cell culture surface area. Schmid and Stoeger (2016) set up a dose-response curve by plotting the particle surface area against the acute inflammatory reaction (PMN influx), attaining a high R^2 of 0.77.

Next to the inflammatory reaction, the influence of compounds on lung surfactant is important as some substances can cause alveolar collapse after inhalation (King, 1982; Schleh et al., 2013). To investigate the influence of airborne substances on lung surfactant, Sørli et al. (2016) established the constrained drop surfactometer (CDS). Their results indicate that the size and the effect of the applied substance on the surface tension of the lung surfactant has an impact on the toxic effect, and can be employed to predict alveolar collapse.

These results from surfactometry as well as the investigation of the inflammatory response induced by substances in the lung indicate that safety investigations of orally inhaled drug products should not be limited to concentration-dependent cytotoxicity tested in a monolayer as in the present work. Nonetheless, the *SAFE* system may assist at early stages of formulation development by relating concentrations in formulations of FDA-approved drug products to concentrations used in human epithelial cell culture experiments. To further expand this approach, inflammatory effects such as the cytokine secretion of macrophages, transport studies to estimate bioavailability, the role of active transporters, and possible interactions with non-cellular barriers (e.g., mucus or surfactant) may be considered as additional endpoints for the safety assessments of orally inhaled drug products.

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Conflict of interest

The authors declare no conflict of interest.

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