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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 the 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 the safety or eventual toxicity in humans. However, the challenge is to link human *in vitro* data 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 concentration 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, allowed to link IC50 data as measured on 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 while the *SAFE* approach allows putting them in the context for eventual use in human pulmonary drug products. Excipients, that are most likely not safe for use in humans, can be early excluded from further pharmaceutical development. The *SAFE* approach helps thus to avoid unnecessary animal experiments.

1 Introduction

Although regulatory agencies, such as the FDA, and the Organization for Economic Co-operation and Development (OECD) encourage the use of *in vitro* assays for the evaluation of new drug formulations, animal testing is still a standard procedure when evaluating inhaled drug products (Silva and Sørli, 2018). The most widely used animals for this purpose are mice, rats, dogs and non-human primates (Pritchard et al., 2003). The costs for animal testing during drug development are between $430m and $1,098m (DiMasi et al., 2016). For comparison, the costs of drug development have increased from $800m to $2,000m, whereby drug development for delivery via inhalation has reached costs of $1,134m per new drug formulation (Adams et al., 2006). Therefore, in 2014 the FDA founded the Critical Path Initiative, which is a project to optimise the costly drug development process. The evaluation of this procedure identified the determination of the safety and efficacy of new drug formulations as a main cost contributor (Woodcock et al., 2008). In addition, the safety aspects account for 24% of clinical study terminations (Harrison, 2016). The FDA Guidance for Industries *`Drug Metabolism/Drug Interaction Studies in the Drug Development Process: Studies In Vitro´* implements *in vitro* studies of the safety and efficacy of potential drugs to exclude any toxic or non-effective drugs directly to reduce the risk of failing during the clinical trials (FDA, 1997). The enormous expense and the ethical need for reducing animal experiments during the non-clinical and clinical trials led to the implementation of *in vitro* tests in the EMA and OECD guidelines, for example to minimise the suffering of the animals (EMA, 2016; OECD, 2000). Next to the financial and ethical aspects, the questionable prediction of human data based on animal experiments is an ongoing discussion (Bracken, 2009; Fröhlich, 2017). According to Albert P. Li, some reasons for this uncertainty might be that animals have different toxic and detoxifying molecular mechanisms and have a different sensitivity when compared with humans. In addition, studies are limited in the investigation of toxic endpoints, the number of tested individuals is restricted, and the studies struggle with dose adaption (Li, 2004). 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).

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However, while there has been progress in *in vitro* skin assays, there is still a lack of adequate cell- and tissue-based *in vitro* methods for other organs, such as the respiratory tract. The OECD was able to draft a guideline in 2018 implementing the certification process and the required qualities for *in vitro* tests which serve the future evaluation of the assessment of human safety (OECD, 2018b). To face this challenge, the development of complex cell culture systems with two or more cell lines for safety evaluation is gaining ground, especially for nanoparticle (NP) deposition in the lung (Chary et al., 2018). Mahmoudi *et al*. reiterates that no systematic data sets for the toxicological assessment of nanoparticles are available, due to non-standardised parameters, different testing systems (cells, animals) and the different types of particles themselves (Mahmoudi et al., 2012). The different endpoints of the obtained *in vitro* data during safety studies – cell toxicity, proinflammatory reactions, translocations of nanoparticles in the tissue and the resulting uptake mechanism to name a few - should be evaluated in a comprehensive way to enable the inclusion in a guideline process (Drasler et al., 2017). Nanoparticles (NP), such as TiO_2, gold and polystyrene particles, were investigated by Rushton *et al.,* who set up a dose response curve by defining the steepest slope as a quantity of the response-metric connected from the *in vitro* and *in vivo* dose-response plots. The *in vitro* assays, which were based on the reactive oxygen species (ROS) secretion of the cells, correlated significantly with *in vivo* observations (PMN recruiting) to a R^2 of 0.81. They proposed a NP hazard scale based on the *in vivo* activity of the NP surface area (Rushton et al., 2014). Nevertheless, 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 evaluation of adverse outcome pathways (AOP´s) with the relating cellular, tissue/organ and organism/population key events after exposure 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 already approved by FDA for some marketed drug products. Nevertheless, even larger datasets, such as obtained by some `omics´-technologies, cannot restrict all limitations of *in vitro* and *in vivo* generated data so far, especially the prediction of adverse effects will be a challenge in the future. (Ghallab and Bolt, 2014).

An important first step for such safety classification might be the LD50 value which is used as a basis for hazard classification (Strickland et al., 2018). Sauer *et al.* investigated 19 chemicals in order to categorize them into four hazard classes predicting their acute inhalative toxicity. This system is based on the comparison of LD50 values *in vivo* with the calculated IC50 values *in vitro* obtained by standardised MTT assays. The resulting comparison between the different cell culture systems and the *in vivo* data allowed the identification of harmful substances (Sauer et al., 2013). In particular, the MTT assay has shown a high sensitivity with regard to the toxicological examinations of various substances by obtaining a simple and reproducible determination of the IC50 value (Scherließ, 2011). However, the aforementioned classification system by Sauer *et al.* already relates to relevant *in vitro* data.

In order to bridge this gap between *in vitro* and human data, a correlation is required. In the present work, 23 excipients with a FDA-approved concentration range (to be considered as safe) were compared with their related *in vitro* IC50 values. The data were used to set up a four-quadrant analysis to classify the safety of 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) were obtained from DSMZ and were 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 obtained from ATCC. The culture medium for the Calu-3 consists of MEM (minimal essential media, 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

For the evaluation of the *in vitro* IC50 values, 23 substances were selected which are listed in Tab. 1 with the corresponding supplier and the solubility in water.

The selected substances are approved for a specified concentration range in parenteral or pulmonary drug application according to the `Inactive Ingredient Search for Approved Drug Products´ provided by the FDA. With this database, a compilation of the approved concentrations is summarized in Tab. 2. A pulmonary-approved concentration was available for citric acid monohydrate, citric acid anhydrous, glycerol, L-ascorbic acid, polysorbate 80, and sodium chloride. If no data for inhalation were given, the parenteral concentration was used.

2.3 Determination of the IC50 value *in vitro*

2.3.1 Preparation of test concentrations

Standardized MTT assays were performed for the evaluation of the *in vitro* IC50. Therefore, several dilutions of the test compounds were required. In the first round of MTT assays the range was narrowed to approximate the IC50.

Therefore, the 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 different cycles of MTT assays were applied for both cell lines. Where the IC50 of an excipient exceeded 10 mg/mL, the concentration was increased 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.

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)

2.3.2 Cell viability measurements

The calculation of the *in vitro* IC50 values is based on a dose response curve, which is constituted by cell viability measurements. MTT assays were performed as follows: 24 h before starting the assay, $2x10^5$cells/ mL of A549 or Calu-3 were seeded in a 96-well plate (Greiner Bio-one, Germany) in a volume of 200 µL of medium. On the next day, the cells were visualized by light microscopy (PrimoVert, Zeiss, Germany) to be sure to verify the epithelial confluence in the 96-well plate of nearly 100%. The cells were washed twice with HBSS and the appropriate concentration in 200 µL of the test substances was applied. After an incubation of 4 h on a shaker with 35 rpm at 37 °C, the cells were washed once with HBSS. The MTT reagent (Methylthiazolyldiphenyl-tetrazolium bromide, Acros organics, USA) was added in a concentration of 0.5 mg/mL per well for 4 h at 37 °C at 35 rpm and protected from light. The 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.

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

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

The software Origin®Pro 2019 (additive, Germany) was used for IC50 calculations based on a dose response curve. Therefore, 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, 3 technical replicates (wells) in 3 independent experiments for each investigated concentration were performed. The IC50 values calculated from A549 were based on n=3 wells per concentration in several experiments to determine the IC50 and narrow the concentrations tested next to it. Details are listed in supplementary information in each graph. Data are summarized as the mean ± one standard deviation (SD). The correlation analysis was performed with the software Origin®Pro 2019. A linear fit without weighting parameters was performed to calculate the Pearson r correlation coefficient.

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

Substance	CAS number	FDA-approved concentration range parenteral	FDA-approved concentration range pulmonal
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%

3 Results

3.1 IC50 of excipients

The cell viability of Calu-3 and A549 was determined for 23 excipients with a standardized MTT assay. After narrowing the range surrounding the IC50, a dose response curve was created with OriginPro 2019. The concentration of 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, and for albumin no determination of the IC50 value on A549 was possible (IC50 >200 mg/mL). The corresponding dose response curves with the IC50 calculation for all tested excipients are listed in the Fig S1 and S2[1].

The *in vitro* IC50 values are listed in Tab. 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.

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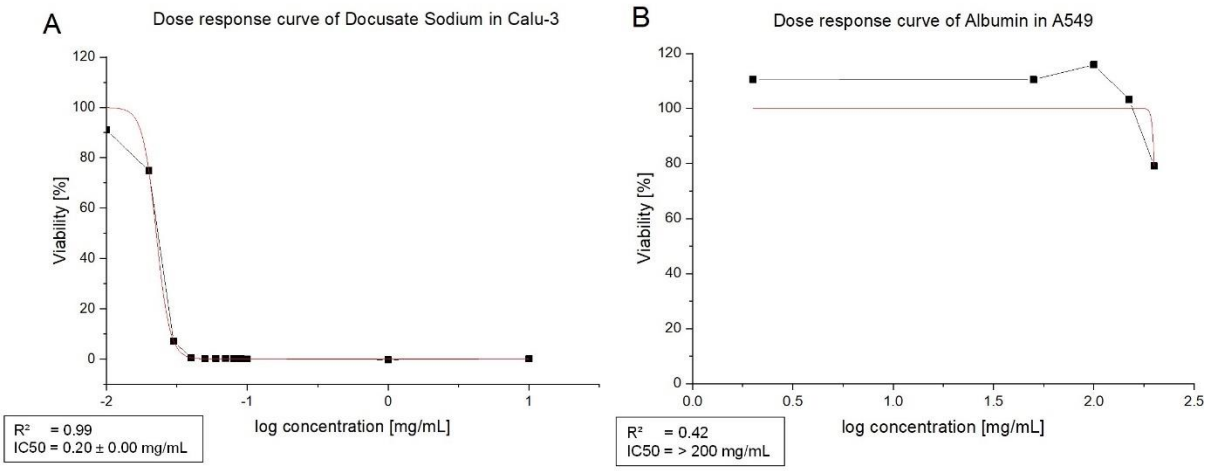

Fig. 1: Exemplary presentation of dose response curve of docusate sodium in Calu-3 and albumin from human serum in A549 for the determination of the IC50 *in vitro*
The graphs were created with OriginPro 2019. The applied concentration is given in mg/mL, whereby the maximal concentration is set to 1 g (undiluted substance). The red line indicates the sigmoidal fit, which is used for the calculation of the IC50 by the software OriginPro 2019. (A) Dose response curve of docusate sodium tested on Calu-3 cells. The calculation of the IC50 is 0.02%. The coefficient of determination (R^2) is 0.99 indicating a usable fit. (B) Dose response curve of albumin tested on A549 cells, the IC50 is not determinable even after exceeding the tested concentration to the maximal solubility of 200 mg/mL.

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 by the software of the IC50 was nonetheless possible. For the remaining 11 excipients, an *in vitro* calculation of the IC50 was successful. In addition, the `Sauer classification´ of the test substances was implemented (Tab. 3). A comparison of A549 and Calu-3 showed that the observed IC50 values are in a similar range but do not correlate in a regression analysis (Fig.S3, Tab.S2[1]).

3.2 Correlation of FDA-approved concentration range vs. *in vitro* IC50

For the evaluation of the linear correlation between the *in vitro* IC50 value and the approved FDA concentration, a regression analysis was performed using the software Origin®Pro 2019.

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, polysorbate 80, polysorbate 80 HX2, polysorbate 20, and sodium chloride were plotted against the approved FDA concentration (Tab. 2) for regression analysis. The percentage ranges of the FDA concentrations given for the pulmonary and parenteral application were included. Fig. 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). Therefore, the IC50 values (Tab. 3) and the FDA-approved concentration (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 likewise obtained by performing a correlation analysis with a 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.

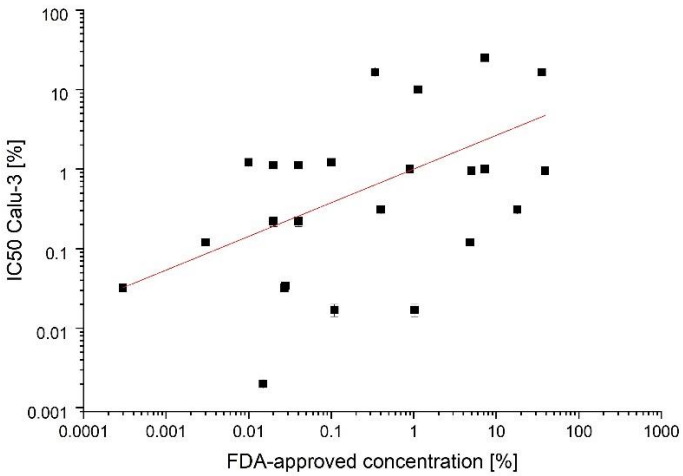

Fig. 2: Regression analysis of IC50 [%] *in vitro* in Calu-3 cells vs. approved FDA concentration range [%].
The concentration of the calculated *in vitro* IC50 values was based on a maximal concentration of 1 g. The approved concentrations were obtained from the `Inactive Ingredient database´ provided by the FDA. A linear regression was performed with the Software Origin®Pro 2019. The values for the 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.

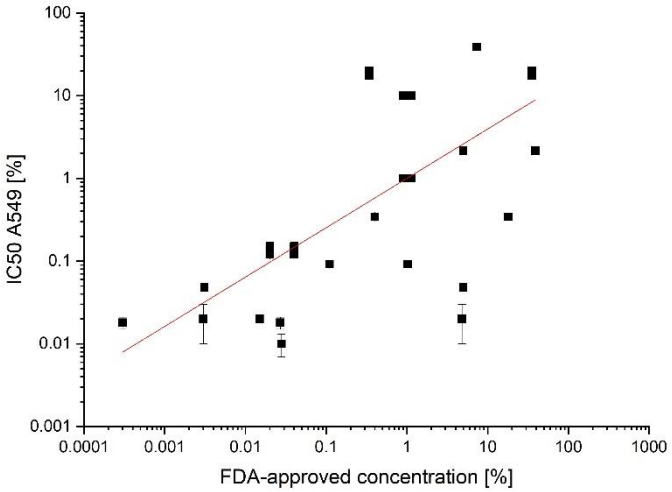

Fig. 3: Regression analysis of IC50 [%] *in vitro* in A549 cells vs. approved FDA concentration range [%]
The concentration of the calculated *in vitro* IC50 values was based on a maximal concentration of 1 g. The approved concentrations were obtained from the `Inactive Ingredient database´ provided by the FDA. A linear regression was performed with the Software Origin®Pro 2019. The values for the 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.

Tab. 3: List of excipients with the calculated IC50 [%] in A549 and Calu-3 cells in comparison to the `Sauer classification´

Substance	CAS number	A549 calculated IC50 *in vitro* [%]	Calu-3 calculated 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

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 a linear regression analysis using the FDA data is that there might be some space for a higher approved concentration as this value cannot be compared with an LD50 derived from animals. However, the knowledge from the Sauer publication can be utilized to consider known hazard effects. By the implementation of the `Sauer classification´ (Sauer et al., 2013) 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 on 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 harmful if inhaled (United Nations, 2017)). The excipients with an IC50 below 0.1% are categorized according to the `Sauer classification´ in classes 1 and 2 (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 `Sauer classification´ of all tested excipients is shown in Tab. 3.

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, a four-class system is obtained. In class 1 there is a consistently high level of the *in vitro* IC50 and a high FDA-approved concentration. For the Calu-3 cell line benzyl alcohol, glycerol, L-proline, sodium chloride, L-arginine and, in the border region, polysorbate- 20 are categorized. These excipients are non-toxic according to the `Sauer classification´. In the A549 cells, class 1 substances have similar values, except polysorbate 20, which is here categorized in class 3-4. Class 2 is in the quadrant to the left, which is

Tab. 4: Line-up of the four-class based `Sauer classification´ given in mg/mL and the consequently 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 tested 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

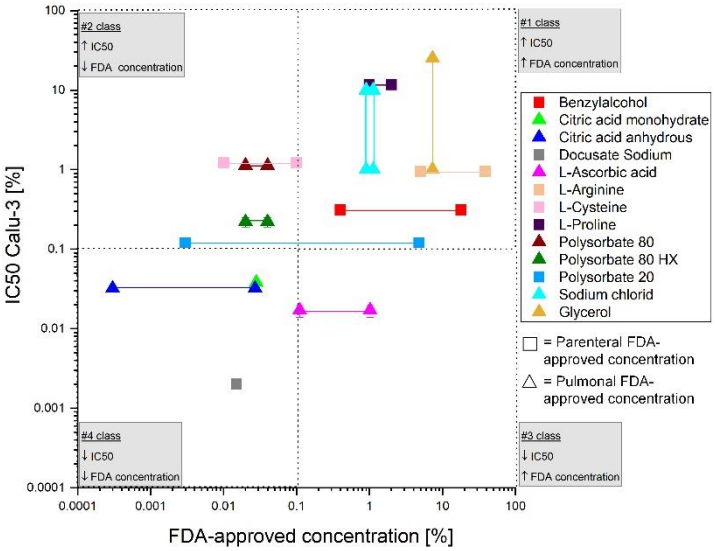

Fig. 4: The IC50 of the excipients tested in the Calu-3 cell line and the FDA-approved concentration ranges can be set up in a four-class system
The diagram consists of the calculated IC50 values plotted against the approved FDA concentrations (squares: parenteral approved concentration, triangles: pulmonary 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.

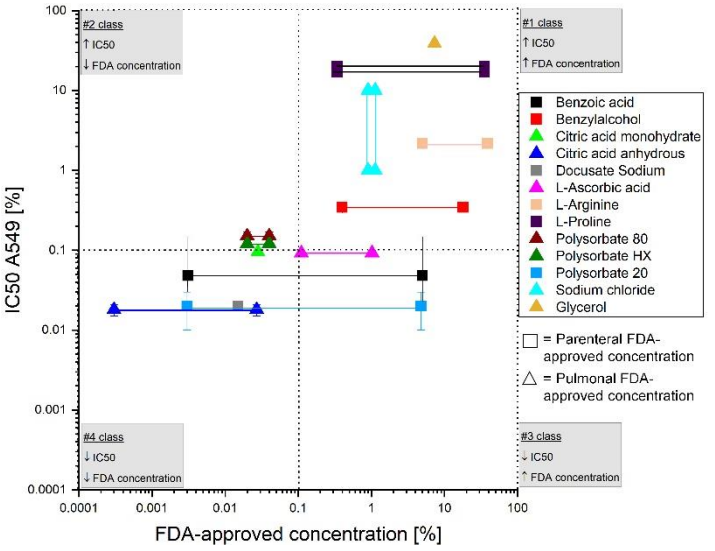

Fig. 5: The IC50 of the excipients tested in the A549 cell line and the FDA-approved concentration ranges can be set up in a four-class system
The diagram consists of the calculated IC50 values plotted against the approved FDA concentrations (squares: parenteral approved concentration, triangles: pulmonary 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.

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 & polysorbate 80 HX2 tested on A549 cells are categorized in 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 it is L-ascorbic acid, benzoic acid and polysorbate 20 which 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 were tested 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 the Calu-3 in Fig. 4 and for A549 in Fig. 5.

3.3.2 Consequences of the four classes for safety assessment of excipients (SAFE)
The *SAFE* classification can help to estimate the likelihood based on *in vitro* IC50 data weather pulmonary administration to humans will be safe or not. This estimation is based on the results presented in Fig. 4 and Fig. 5. The concentration of 0.1% (which indicates problems with the animal toxicity study at the end of the preclinical phase) forms the centre of the coordinate system and is the dividing line for the resulting classification (Fig. 6).

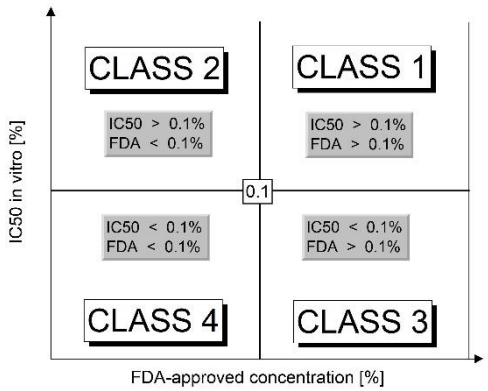

Fig. 6: The *SAFE* classification
Divided into 4 classes, the *SAFE* gives an indication of the categorization of the *in vitro* IC50 value to the FDA-approved concentration. Arrangement of the 4 classes: Class 1: IC50 is higher 0.1%, the correlated FDA concentration is higher 0.1%; Class 2: IC50 is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

An increased IC50, which is above 0.1% (classes 1 and 2), is worth considering for further development. A lower IC50 under 0.1%, which is here referred to classes 3 and 4, is not recommended for further developments. The target class 1 implements a high IC50 and a high FDA-approved concentration, making it the most appropriate class to submit for further testing and potential use in humans. Most tested excipients are classified in this non-toxic category and no safety issues should be expected during the 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 `Sauer classification´, cytotoxicity is obvious, and therefore considering of such compounds for further development steps are maybe 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 eventually obtaining FDA approval by using those 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 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 bindings, 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 for a dose dependency and comparison with *in vivo* data was possible (Eixarch et al., 2010). The present work is an attempt to break down the complexity of the physiological aspects of the lungs to gradually approach an *in vitro* test strategy for orally inhaled drug products. For this purpose, cytotoxicity towards pulmonary epithelial cells, which can support formulation development, was primarily addressed. The need for an *in vitro* testing strategy for predicting of the respiratory toxicity of chemicals and drug products has become ever more present. In keeping with the established guidelines for *in vitro* skin sensitizers (for example OECD Test No. 442E: *In Vitro* Skin Sensitization), it should be possible to develop such a test system for the lung. To establish a respiratory *in vitro* testing strategy, one initial step is a classification system for the safety aspects of chemicals and drug products – as many recently published articles show. Through an *in vitro* correlation of the IC50 values to the GHS classification of 19 substances, an *in vitro* hazard classification system could be established (Sauer et al., 2013). We slightly modified the experimental setup of Sauer *et al*. by adjusting the incubation period to 4 hours, in order to consider the exposure period mentioned in the OECD acute inhalation toxicity guideline 436 (OECD 2009; Sauer et al. 2013). We applied the test compounds in a salt buffer for 4 hours in DMEM instead of 24 hours. This should reduce misleading results due to cell proliferation and the influence of the incubation medium. The impact of this modification, the selection of the cell types, the incubation period and the selected threshold are certainly worth be discussed. Sauer et al. have tested 3 cellular systems with different incubation periods (A549 – 24 hours; EpiAirway™- 3 hours; MucilAir™ system – 24 hours). 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 GHS category ≤ 3. 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 rather clear indication. We recommend when developing a new drug product also to test pro-longed incubation periods and other cell types when applicable. However, the `Sauer classification´ forms the basis for the *in vitro* IC50 limiting value of 0.1% of the SAFE classification.

When developing the SAFE classification concept, the toxicological considerations were mainly based on the *in vitro* IC50 evaluation. To obtain an IVIVC in a first step, a connection with the LD50 *in vivo* based GHS classification should be possible. Therefore, the GHS classification was compiled for the tested excipients (Tab. S1[1]). A large data gap was noted during the collection of the pulmonary LD50 values, so that no effective and direct correlation of the IC50 values to the *in vivo* data 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)). A direct comparison to the GHS classification based on LD50 data does not work for pharmaceutical excipients as most of them are classified as safe (GHS class 4/5, see Tab. S1[1]). The tested excipients were toxicologically harmless via the oral application route, therefore the acute toxicity oral classification by the GHS is Class 4 or 5. For the pulmonary classification the exposure route is divided into gases, vapours, dusts and mists (United Nations, 2017). For these application forms, no increased GHS classes were obtained. For gases of benzyl alcohol, GHS Class 1, docusate sodium, sodium chloride, and polysorbate 20 Class 2 were provided. This use of different routes of administration *in vivo* faces the *in vitro* inability to reach a standardized comparison. With the aim of achieving a better IVIVC, even more complex cell culture systems have been applied (Fizeșan et al., 2018). By testing the cytotoxicity of inhaled drug products in a commercial available human 3D cellular model of the lung (MucilAir), Sivars *et al*. could implement a good correlation of *in vivo* toxicity data to the respiratory toxicity *in vitro* by using different methods to evaluate the cell barrier integrity, cell viability, ciliary beating frequency (CBF), mucociliary clearance, and the resulting cytokine release (Sivars et al., 2018). In the mentioned study, MucilAir was cultivated under serum-free conditions avoiding the use of FCS, which is associated with pain and suffering for the animals. The amount and type of serum proteins is of particular importance as they can influence safety aspects of nanomaterials (Drasler et al. 2017; Moore et al. 2015). From this point of view FCS should be replaced 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 in the microfluidic systems. Huh et al. 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. More complex systems such as co-cultures or chip-based systems are challenging to validate (Huh et al., 2011; Dipasri et al., 2016). Therefore, as in the present work, the complexity might be reduced so that a stepwise *in vivo* correlation is feasible. The *in vitro* test strategy we addressed in this work might be extended and combined with further specific methods (e.g. surfactometry) afterwards and summarized into a safety picture of the exposed compound. Such integrated test strategies for the *in vitro* prediction of acute inhalation toxicity were discussed 2018 in the workshop `Alternative approaches for acute inhalation toxicity testing to address global regulatory and non-regulatory data requirements´. Clippinger et al. summarised here the current state of inhalative *in vitro* technologies as well as the missing criteria 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 (Clippinger et al. 2018b).

The toxic effects of substances in the lung have different initial points, such as airborne concentration, size of particles, solubility in the lung tissue, 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 building up a tetraculture of A549, HMC-1, THP-1, and EA.hy 926, and by using the Vitrocell® CLOUD system, Klein et al. could show an over-prediction of inflammatory responses under submerged conditions (Klein et al., 2013). This result could be an explanation for the high FDA-approved concentration and low IC50 value (SAFE class 3). To avoid this overestimation, various 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 in consideration of ongoing discussions on dosimetry (Wiemann et al., 2018; Schmid et al. 2017) and experimental setups (Kong et al. 2011). The dosimetry is an important aspect of focusing the deposition of particles or substances in the alveolar region. Donaldson et al. obtained a high IVIVC by expressing the dose in A549 cell culture surface area and the subsequent importance of the usage of the particle surface area metric (Donaldson et al., 2008). Schmid et al. set up a dose-response curve by plotting the particle surface area against the acute inflammatory reaction (PMN influx), implementing a high R^2 of 0.77 (Schmid and Stoeger, 2016). Next to this proven inflammatory reaction the influence on lung surfactant is important for avoiding alveolar collapse after inhalation of substances (King, 1982; Schleh et al., 2013). To investigate the influence of exposed substances to the lung surfactant, Sørli et al. established the constrained drop surfactometer (CDS). Their results indicate that the size and effect of the applied substances on an increased surface tension of the lung surfactant has an impact on the toxic effect, resulting in alveolar collapse (Sørli et al., 2016). By examining the surfactometry as well as the inflammatory response after application of the substances into the lung, it becomes clear that the safety investigations of orally-inhaled drug products should not be limited to one aspect of the concentration-dependent cell toxicity tested in 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 cultures experiments. To further expand this approach, inflammatory effects such as the cytokine secretion of macrophages, transport studies to estimate bioavailability, active transporter and possible interactions with non-cellular barriers (e.g. mucus or surfactant) may be considered as additional endpoints for the safety assessments of oral inhaled drug products.

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Conflict of interest
The authors declare no conflict of interest.

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