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The MTT assay as tool to evaluate and compare excipient toxicity *in vitro* on respiratory epithelial cells

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ABSTRACT

There are not many excipients already approved in drug products for the use in the respiratory tract. In this study, a rapid *in vitro* screening procedure to assess and compare acute toxicity of soluble excipient substances on respiratory epithelial cells utilising the Calu-3 cell line is presented. The test substances are either dissolved in HBSS + HEPES buffer or are directly applied to the cellular surface. After 4 h incubation, the substances are removed and the cell viability is assessed using an MTT assay. The tested excipients include polysorbate 20 and 80, lactose and povidone 30 as well as glycerol and propylene glycol as examples of excipients already being used in formulations for application in the respiratory tract. These substances are sorted according to their toxic effect and new excipients not yet used in the respiratory tract like HPMC can be classified in this scheme. With this, besides information from systemic toxicity tests, a first valuation of the acute toxic effect of the substance on respiratory epithelial cells is gained. This can aid in the choice of new excipients being necessary for modern respiratory formulations comprising new active compounds as biomolecules or new delivery strategies such as sustained or prolonged delivery.

1. Introduction

The application of antiasthmatic drugs to the respiratory tract has a long history and is well established. Those drugs mainly have a local effect in the lung. In this case, a systemic effect would be a side effect and has to be avoided. Furthermore, there are some drugs being taken up via the respiratory tract inducing systemic effects like nicotine and anaesthetic gases (von Wichert and von Wichert, 2006). Increasing interest has been put on the area of systemic uptake of biomolecules via the pulmonary route and there are already some promising examples like insulin described in literature. Especially the rising number of biotherapeutic agents like peptides and proteins as potential drug candidates evokes interest in using the systemic uptake via the respiratory tract (Agu et al., 2001). Those drugs are easily degraded by enzymes in the gastrointestinal tract and, therefore, a parenteral formulation with all its disadvantages with respect to stability and compliance is often needed.

From the biopharmaceutical aspect, it is advantageous to use a non-oral route for the application of biomolecules as absorption might be higher due to higher substance stability. But unlike small molecules such as β-agonists, corticosteroids or antibiotics, most biotherapeutic agents are macromolecules normally having poor absorption rates. The respiratory route has been shown to reach a quite high bioavailability for peptides when compared with other common application routes like oral, rectal or dermal application (e.g. desmopressin; Fjellestad-Paulsen et al., 1993). For insulin, the bioavailability can approach or exceed 100% relative to subcutaneous injection (Patton, 1996) and also other macromolecules like interferon-α or the human growth hormone have been shown to be taken up efficiently via the lung.

From the formulation aspect, biomolecules are often more challenging to formulate as the ternary structure might be essential for the effect and needs to be maintained, especially when formulating a peptide or protein. The formulation has to protect the molecule while preserving its function and mediating delivery and uptake (Gonda, 2007). These prerequisites require a sophisticated choice of excipients and formulation strategies. There are only few excipients which are registered for the use in the respiratory tract like sugars, some solvents and a few surface active substances and the will to register new substances as excipients is very restricted due to the high costs of the toxicity studies demanded by the authorities (Steinberg et al., 1996; DeGeorge et al., 1997; Baldrick, 2000). Due to the very limited repertory of excipients for the respiratory administration route, it is extremely difficult to formulate modern drugs or to create functional formulations with mucoadhesiveness or sustained release. To formulate new pharmaceuticals for the respiratory tract, a broader tool kit of excipients is needed.

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To evaluate the potential of a new excipient, it is not only indispensable to assure the functionality with respect to the formulation, but it might also be valuable to get some information about the toxicity of such a substance in advance. Consequently, it is considered helpful to set up *in vitro* studies to screen the toxicity of novel and new excipients prior to the pre-clinical assessment in animal studies (Rothen-Rutishauser et al., 2008) and this question has extensively been discussed in a FRAME workshop (BéruBé et al., 2009). A cell culture model can be used as *in vitro* model for toxicity screening in a living system. Commonly used cells for toxicity studies are easy to cultivate, fast growing and sensitive to toxic irritation, such as fibroblasts (Gupta and Gupta, 2004) or Chinese hamster ovary (CHO) cells (Kaye et al., 2009). For more tissue specific toxicity studies, specialised cell models can be used like the EpiDerm™ skin model for the *in vitro* evaluation of skin irritation (MatTek, Ashland, MA, USA). There are many pathways for inducing a toxic effect; therefore, different detection techniques can be used. Besides a global live-dead differentiation as possible with live/dead staining, there are methods detecting membrane disintegration, enzyme malfunction or the induction of apoptotic pathways. The mechanisms of cellular toxicity can be diverse and may also be different according to the exposure time. In preclinical safety assessment, it is distinguished between acute and chronic effects (immediate and long-term effects on the exposed individual) as well as effects on the following generation (hereditary effects). Among the mechanisms of acute toxicity, the direct cell membrane damage is the most pronounced mechanism resulting in cellular leakage. Further on, the cellular function may be restrained due to enzyme malfunction or disorganisation. Another, less acute, effect is a change in translation, transcription or a genetic effect resulting in a change in the cellular equipment. With regard to possible substance effects, cellular toxicity may take place by direct membrane interaction such as membrane solubilisation or disintegration as expected from surfactants. Enzyme malfunction may be caused by protein precipitation or degradation, whereas unspecific cellular leakage can also be caused by pH shift or hyperosmolarity. All these effects lead to a reduced cellular viability which can be taken as an indicator for acute toxicity and is easy to measure.

The Calu-3 cell line, a cell culture model of the respiratory epithelial cells, has already been evaluated for the use as tool to screen respiratory drug delivery (Foster et al., 2000). Calu-3 cells can be cultivated submersed under liquid or in Transwell® systems where the cells are cultivated on membrane inserts. The formation and integrity of the monolayer can then be followed by measuring the transepithelial electrical resistance (TEER), which can serve as a very sensitive marker for cellular damage in a Transwell® system. The cells are fed from the basolateral compartment, whereas the apical compartment can be emptied and the cells get direct air contact resulting in an Air-Interface-Culture (AIC). The cells then secrete mucus on the apical surface and with this resemble more the physiological system where the epithelium in the respiratory tract has a direct contact to air (Grainger et al., 2006). In the herepresented work, the Calu-3 cell line is used for the evaluation of the acute toxicity of excipients commonly used in respiratory formulations.

2. Aim of the study

The aim of the study was to compare the toxicity of known and novel excipients of different functionalities for the use in formulations applied on the respiratory epithelium by means of an *in vitro* cytotoxicity assay. For this, different *in vitro* assays are evaluated for their use as a screening test for cellular damage and toxicity on the respiratory epithelial cells represented by Calu-3 cells. The results of known excipients can then be compared to the toxicity of further excipients not yet registered or approved for use in the respiratory tract. So, it is possible to estimate the irritant/toxic potential of new excipients in relation to other excipients. With this, the excipient choice for biomolecule formulations can be more specific, so that the formulation development already utilises substances being less toxic. This leads to a more rational formulation development, because the formulation will probably not be dismissed due to toxicity problems later on.

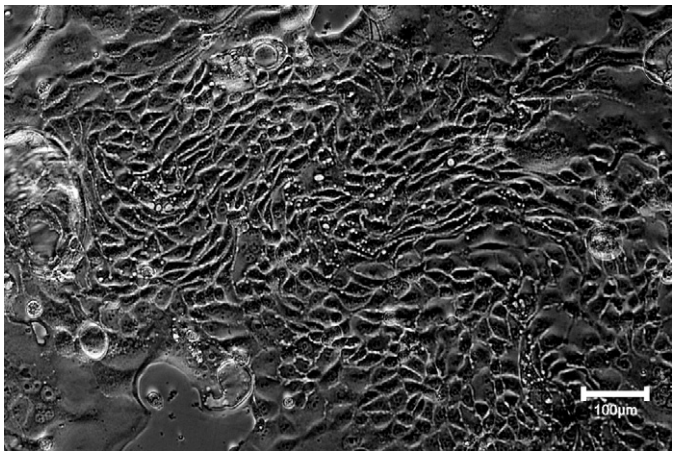

Fig. 1. Typical microscopic image of the cellular layer of Calu-3 cells in culture (bar = 100 µm).

3. Materials and methods

3.1. Cell line

The Calu-3 (HTB-55) cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA); passage number 37–50 has been used for all toxicity studies. The Calu-3 cell line is an adherent cell line derived from a bronchial adenocarcinoma (25 years old male Caucasian). The cells easily form a monolayer culture and can be cultivated in flasks, 96-well-plates and on membrane inserts. Calu-3 cells have shown to be a suitable model for the respiratory tract (Steimer et al., 2005). The growth medium used for the cells is a Minimum Essential Medium (MEM) with Earle's salts supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) Penicillin–Streptomycin, 1% (v/v) non-essential amino acids (100×) and 1% (v/v) sodium pyruvate (100 mM) (all from Biochrom AG, Berlin, Germany). The cells are cultivated at 37 °C at 95% rH and 5% CO_2 supplementation. For subcultivation, the cells are grown in cell culture flasks with 75 cm^2 bottom (Greiner bio-one, Frickenhausen, Germany), from where they are trypsinated (with trypsin/EDTA 0.25%/0.02%; Biochrom AG, Berlin, Germany) at a confluence of about 80%, diluted and seed to 96-well-plates (Biochrom AG, Berlin, Germany) at a cell number of 1.5×10^5 cells/ml. Each well is filled with 200 µL cell suspension resulting in a cell number of 3×10^4 cells per well. The cells are allowed to form a monolayer for three days before the toxicity test is started. A typical microscopic picture of the cell layer is displayed in Fig. 1. For cultivation in an Air-Interface-Culture, the cells are transferred to 12-well-Transwell® plates (Corning B.V. Life Sciences, Amsterdam, The Netherlands) at a cell number of 4×10^5 cells per well. In the Transwell system, the formation and integrity of the monolayer is followed by TEER (transepithelial electrical resistance) measurements (EVOM, WPI, Sarasota, FL, USA). In the Air-Interface-Culture (AIC), the medium in the apical compartment is removed after one week and the cells are grown at direct air contact. The production of mucus on the apical membrane is confirmed by mucus staining (with alcian blue) prior to the experiment.

Table 1
Tested excipients and examples of marketed products comprising the respective substance (Rote Liste, 2009).

Excipient	Example of product with the respective excipient	Marketing authorisation holder
Sugar/sugar alcohols		
Lactose	Foradil® P	Novartis Pharma
Glucose	Pulmicort® Topinasal® 64 μg	AstraZeneca
Sorbitol	Allergocrom® nasal spray	Ursapharm
Surfactants		
Polysorbate 20	Sanasthmax® 400 μg/1 ml solution for nebuliser	Chiesi
Polysorbate 80	Pulmicort® 0.5 mg/2 ml suspension for nebuliser	AstraZeneca
Solvents		
Glycerol	Budiair® FCKW-free 0.2 mg	Chiesi
Macrogol 400	Syntaris® nasal spray	Dermapharm
Propylene glycol	InfectoKrupp® Inhal	Infectopharm
Ethanol	Apsomol® N Aerosol	Farmasan
Polymers		
Hypromellose (HPMC 50)	Allergodil® nasal spray	MEDA Pharma
Carmellose-sodium (30)	Beclomet® 100 μg nasal	Orion Pharma
Povidone K30	CromoHEXAL® N	Hexal
Gelatin	As capsule material in FormoLich® 12 μg	Winthrop
Povidone K25	n.a.	
Macrogol 1500	n.a.	
Macrogol 4000	n.a.	
Macrogol 35000	n.a.	
Others		
Sodium chloride	Sultanol® instant solution for nebuliser	GlaxoSmithKline
β-Cyclodextrine	n.a.	

3.2. Excipients

The examined excipients are listed in Table 1 and were purchased from common suppliers. The excipients are mostly tested in solution. For this, the excipient is dissolved in Hanks buffered salt solution (HBSS; Biochrom AG, Berlin, Germany) supplemented with 30 mM HEPES-buffer (Biochrom AG, Berlin, Germany) in the highest used concentration (stock solution). All further concentrations are made from this stock solution by diluting with HBSS + HEPES buffer. The analysed concentrations are selected to cover a range from 0 to 100% viability for each substance in order to calculate an LC_{50} value subsequently. If the cells are cultivated in an AIC, the excipients can be applied to the cellular surface in different masses without dissolving.

3.3. Toxicity assays

3.3.1. MTT assay

The toxicity of the excipients can be assessed using the well known MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) test as first described by Mosmann (1983). The test has been modified several times since then (Hansen et al., 1989) and was validated in the lab at the beginning of this study. Viable cells are able to reduce the yellow MTT under tetrazolium ring cleavage to a water-insoluble purple-blue formazan which precipitates in the cellular cytosol and can be dissolved after cell lysis, whereas cells being dead following a toxic damage, cannot transform MTT. The reaction is mediated by dehydrogenases – enzymes associated with the endoplasmatic reticulum and the mitochondria (Fotakis and Timbrell, 2006). First, the medium is removed from the cells in 96-well-plates by gentle sucking and 200 μL of the excipient solution are pipetted to each well (quadruple determination), which is then incubated at 37 °C for 4 h. Afterwards, the excipient solutions are removed and 25 μL MTT solution (5 mg/ml in HBSS + HEPES) is added to each well and is incubated for 2 h. Subsequently, all cells are lysed with 100 μL lysis solution (5% SDS in DMF:water 50:50, pH 4.7) per well. A solution of 5 mM SDS (sodium dodecyl sulphate) in HBSS + HEPES is used as positive control (0% viability); negative control (100% viability) is HBSS + HEPES. In the Transwell® system, the medium in the apical compartment is removed by sucking, afterwards 0.5 ml of excipient solution is incubated on the cells for 4 h. After the excipient solution is removed, the basolateral compartment is emptied as well and the MTT solution is given to both compartments (0.1 ml upper and 0.5 ml lower) for 2 h incubation. After the MTT is removed from the basolateral compartment, 0.5 ml lysis solution is given in the apical compartment for overnight cell lysis. If the excipients are tested in the AIC, they are directly dispersed on the apical membrane. After 4 h incubation the remnants are removed by gentle washing with HBSS + HEPES prior to the addition of MTT solution. Detection and quantification of the formazan crystals is performed by a multiwell plate reader (Thermo Spectra III Reader with software easyWIN-fitting, V6.0a, Tecan, Austria) at 570 nm (reference wavelength 690 nm).

3.3.2. Neutral red assay

The neutral red (NR) assay is based on the principle that living cells are able to take up the cationic dye neutral red (3-Amino-7-dimethylamino-2-methylphenazine hydrochloride) and to store the dye at the negatively charged (inner) surface of lysosomes, whereas dead cells lose this characteristic. The extent of colouration is a measure for cell viability. After medium removal from the 96-well plate and 4 h incubation with 200 μL excipient solution (quadruple determination) in the incubator, the excipient solutions are removed again and 100 μL of the staining solution (50 μg/ml neutral red in HBSS + HEPES buffer) are added to each well. After 3 h incubation at 37 °C, the dye solution is removed from the cells and they are lysed with 100 μL lysis solution (50 ml ethanol + 1 ml acetic acid to 100 ml double-distilled water) per well. Quantification of the neutral red amount is performed photometrically with a multiwell plate reader at 520 nm (reference wavelength 690 nm). A solution of 5% Triton X 100 (Sigma–Aldrich, Steinheim, Germany) in HBSS + HEPES is used as positive control (0% viability), HBSS + HEPES buffer is used as negative control (100% viability).

3.3.3. LDH assay

When cellular irritancy takes place, the cellular membrane often is the first to be damaged (especially from surface active components) resulting in a leak in the membrane. With this, cytosolic components can leak from the cells to the surrounding medium. This is also true for lactate dehydrogenase (LDH), an enzyme of the cytosol. The LDH assay detects this LDH leakage: the higher the LDH in the medium, the more damaged is the cell. For detection, the enzymes' ability to convert lactate to pyruvate is utilised. With this, NAD+ is reduced to NADH/H+. In a second reaction step, the catalyst PMS (phenazine methosulphate) transfers H/H+ from NADH/H+ to the yellow tetrazolium salt MTT which is reduced to a purple-blue formazan and which can be quantified photometrically as described before. After medium removal from the 96-well plate and 4 h incubation with 200 μL excipient solution (quadruple determination) at 37 °C, 100 μL of the supernatant is transferred to a new 96-well plate. 100 μL of a solution of 7.5 mM NAD, 1.6 mM PMS and 3.9 mM MTT in lactate–Tris buffer (2.4 g Tris (2-amino-2-(hydroxymethyl)-propan-1,3-diol) in 50 ml water plus 50 mM lactic acid 30% to 100 ml water) is added to each well and the plate is incubated at 37 °C for 30 min, allowing the conversion of MTT to the blue formazan. Afterwards, the reaction is stopped by adding 50 μL 0.1 N acetic acid to each well and the formazan is quantified photometrically with a multiwell plate reader

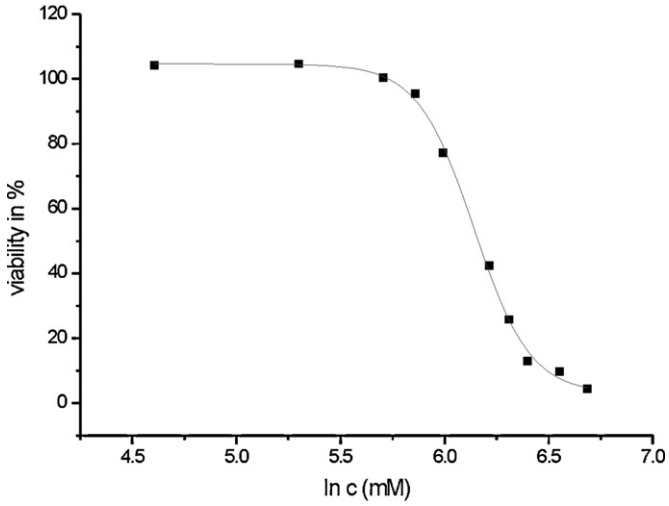

Fig. 2. Example of sigmoidal curve fit (ln *c* against viability in %), data from lactose, calculated from $n = 4$.

as stated before. A solution of 5% Triton X 100 (Sigma–Aldrich, Steinheim, Germany) in HBSS + HEPES is used as positive control (0% viability), HBSS + HEPES buffer serves as negative control (100% viability).

3.4. Data analysis

In the case of MTT and NR assay, the absorption intensity is a measure for the amount of living cells, whereas it is the opposite in the case of the LDH assay. The relative cellular viability is calculated from the absorbance values (as a percent of the negative control). All absorptions were converted to viability results individually; the mean value of four determinations was then taken for further calculations and analysis. LC_{50} values were calculated from the mean results by plotting the natural logarithm (ln) of the concentration against cellular viability. The resulting curve was fitted by a sigmoidal fit (Boltzman fit) utilising the analysis function of Origin 7 (Origin 7G SR1, V7.0303, OriginLab Corporation, Northampton, MA, USA) and the corresponding concentration at a value of 50% viability was calculated from the sigmoidal fit parameters and is expressed as LC_{50} (Fig. 2: example for curve fit). If any parameter of the sigmoidal fit has a deviation greater than the parameter value due to lack of fit, the calculation of LC_{50} is not performed. A comparison of the toxic effect is performed in different ways: If an LC_{50} calculation is applicable, then the LC_{50} value is either indicated by the percentage (w/w) where 50% of the cells are viable, by an amount in mg substance per cm^2 or by the amount in mM, if the substance has a specified molecular weight. Otherwise the percentage (or mg/cm^2 or mM) where no adverse effect on the cells is observable (>80% viability) is used as a measure.

4. Results and discussion

4.1. General variables and choice of toxicity assay

For general test validation, different variables of the toxicity assay such as method of medium removal (by sucking or by decanting), cell number (1×10^4 or 3×10^4 cells per well), positive and negative control substances and washing steps were evaluated with respect to data processability and reproducibility. For comparison with the literature data, all preliminary experiments were performed in 96-well plates with β-cyclodextrine using the MTT assay, where the toxic effect on Calu-3 cells has already been described in literature (Matilainen et al., 2008). The assay showed a good comparability with the literature data as they found a viability of 34.5% (±7.3%) for 15 mM β-cyclodextrine and the here presented assay showed 35.5% (±4.3%) viability. It was found that the general parameters as stated in the above methods section are best for the determination of acute toxic effects. In order to assess the best suited assay, different methods for the determination of cellular toxicity were evaluated. These were the MTT assay, the neutral red assay and the LDH assay. Whereas MTT and NR assay are based on the ability of living cells to take up the dye and store it in lysosomes (in the case of NR) or to convert it enzymatically to another colour (in the case of MTT), the LDH assay is based on the detection of an enzyme leakage in the supernatant medium following cellular membrane damage. All three tests have been described for the evaluation of toxic effects on cell culture based systems (Fotakis and Timbrell, 2006). Within the Calu-3 test system, the MTT assay exhibits the highest sensitivity, being about three times as sensitive as the NR assay. This is due to the different mechanisms of the two tests. Whereas the neutral red assay detects complete loss of cellular function resulting in an inability for electrostatic storage of the positively charged dye to the negatively charged lysosome, the MTT assay targets the catalytic function of dehydrogenases, which might be influenced from toxic effects much easier. Nevertheless, it has to be kept in mind, that the MTT assay is based on a reduction reaction of the dye resulting in a change in colour. This reduction is mediated enzymatically, but other reductive substances may interfere with this mechanism. Therefore, all substances were checked for erroneous signals in the assay. The LDH assay did not come up with reasonable results and was therefore regarded as insufficient for this test system. This result can be explained by the test procedure: if a substance causes cellular leakage, LDH is released in the supernatant, which in this case is the substance solution itself. If the test substance is able to interact with the enzyme, as likely e.g. in the case of surfactants, the enzyme might be degraded and its function cannot be detected. This leads to the erroneous assumption that no LDH was released due to an intact membrane. This drawback has also been described in the literature (Weyermann et al., 2005) and is observed in the test system, where even substances resulting in a visible damage of the cells, give no LDH leakage signal. In conclusion, the MTT assay was regarded as the best suited assay for evaluation of acute excipient toxicity in the Calu-3 model.

4.2. Influence of osmolarity and pH

As cellular viability strongly depends on the medium conditions such as pH value and osmolarity, the influence of these parameters on cellular viability was assessed as well. For this, aqueous solutions containing different amounts of NaCl to achieve different osmolarities at different pH values (adjusted with 1 N HCl or 1 N NaOH, respectively) were prepared and tested on the cells. It was concluded, that the maximum viability of the Calu-3 cell line is maintained at a pH of 6–7 and an osmolarity of 290–390 mosmol (Fig. 3). In order to keep these conditions while stressing the cells with the test substances, all solutions were subsequently prepared with HBSS + HEPES buffer, itself having a pH of 7.2 and an osmolarity of 348 mosmol. HBSS has an optimal salt selection to maintain the cellular viability. The addition of 30 mM HEPES results in an enhanced buffer capacity and with this helps to keep the pH value constant. Further on, high concentrations of the tested substances result in hyper osmolar solutions and with this may reduce cellular viability unspecifically. This effect cannot be excluded, but it is regarded to be part of the overall toxic effect of substances when present in high concentrations, as it might happen in the respiratory tract as well. Therefore, the excipient test solutions were used as is.

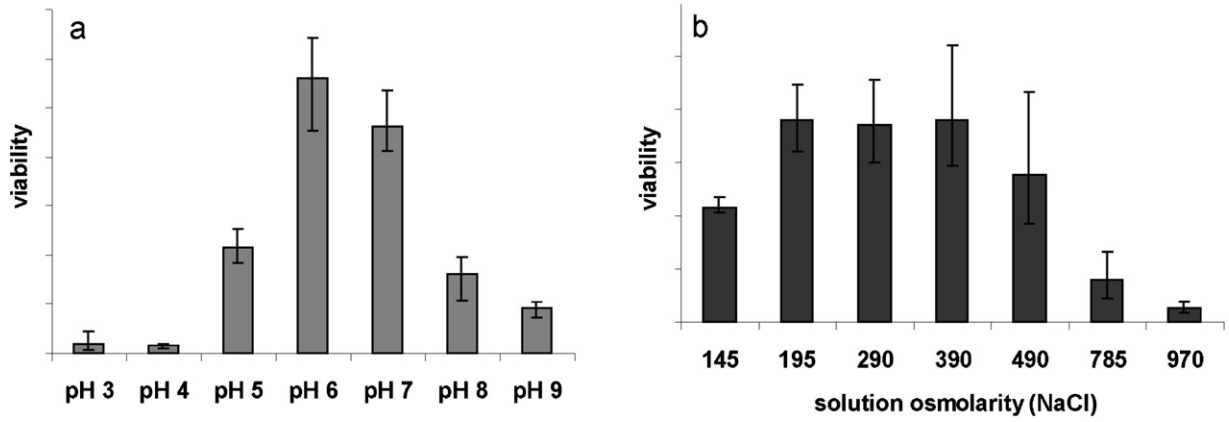

Fig. 3. Viability of Calu-3 cells after 4 h treatment with solutions of different pH or osmolarities. (a) Viability in dependency on pH ($n = 4$, pH adjusted buffer with an osmolarity of 300 mosmol). (b) Viability in dependency on osmolarity ($n = 4$, osmolarity adjusted NaCl solution with pH of 6.8). Error bars are min–max values.

4.3. Toxicity of respiratory excipients

All substances were tested in three to five concentrations for pre-evaluation of the concentration range, which should be tested in detail. The tested concentrations should cover the range from 100% viability to 0% viability in order to be able to calculate an LC_{50} value. After this screening, a range of concentrations for each substance was tested. According to the optical density of the individual wells on the plate, the % viability was calculated. Ideally, the concentration range covered 0–100% viability, as shown for lactose in Fig. 4. It is assumed, that the ln concentration–viability curve follows a sigmoidal curve as already shown in Fig. 2. All concentrations were tested in quadruplicate and the mean value is used for further calculation. It can be seen from the data by the max–min values in Fig. 4, that there was some deviation in the results, but this is due to the living system and was regarded as normal. Some substances could not be tested in a concentration high enough to achieve viability values close to 0%, therefore a calculation of LC_{50} was not applicable in these cases. If LC_{50} calculation was not applicable (in the case of very toxic or very nontoxic substances), the concentration, where no toxic reaction was observed (>80% viability) served as a measure for toxicity estimation. The examined excipients can be divided into different groups due to their functionality. When using an excipient is being considered, the choice is normally guided by the excipients' function. Therefore, it is meaningful to compare excipients of the same functionality. The examined substances were classified into the following groups

(a) surface active substances/solubilisers;
(b) solvents;
(c) polymers (as mucoadhesives/matrix);
(d) sugars/sugar alcohols as stabilisers for biomolecular formulations;

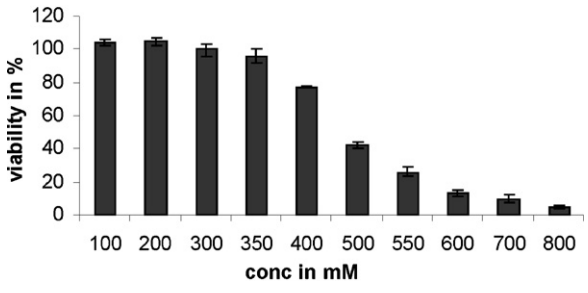

Fig. 4. % viability of Calu-3 cells after 4 h incubation with lactose in different concentrations ($n = 4$, error bars represent min–max values).

where some excipients may serve in more than one function. There are further excipients in respiratory formulations such as preservatives, flavours and substances to set the pH value of a solution or to increase the buffer capacity, but these were not included in the test.

Within the described groups, it could be assumed that the surface active components show the highest toxicity due to a possible interaction with the cellular membrane. This interaction can be utilised if the substances are used as enhancers (Davies and Illum, 2003), but it often goes along with irritancy and toxic effects. In the MTT assay, some substances showed a clear concentration dependent toxicity, from which an LC_{50} value could be calculated (Table 2), such as the two polysorbates Tween 20 and Tween 80 (Fig. 5). Both are already used in respiratory formulations although they showed a high toxicity with an LC_{50} of 0.9 mM (polysorbate 20) and 8 mM (polysorbate 80). Unlike the behaviour of polysorbates with respect to solubilisation, where a longer fatty acid chain results in a lower concentration needed for the same solubilising effect, the longer chain length entails a lower toxicity and a lower increase in toxicity with increasing concentration.

If the toxic threshold and the concentration needed to achieve total cellular death are compared, the resulting interval can be described as safety index. It is a measure of the rate of increase in toxicity with an increase in concentration. If this interval is big, an increase in concentration does not result in a remarkable increase in toxicity. This "safety buffer" is advantageous for use in respiratory

Table 2
Comparison of LC_{50} values in mM of the tested excipient, grouped due to their functionality (n.a. = not applicable due to high viscosity or no explicit MW).

Excipient group	Substance	LC_{50} in mM
	NaCl	515.79
Surface actives	Polysorbate 20	0.88
	Polysorbate 80	7.95
Solvents	Macrogol 400	553.53
	Ethanol	771.05
	Glycerol	2800
	Propylene glycol	3350
Polymers	HPMC 50	n.a.
	CMC-sodium	n.a.
	Gelatin	n.a.
	Povidone K25	n.a.
	Povidone K30	n.a.
	Macrogol 35000	9.04
	Macrogol 4000	70.28
	Macrogol 1500	182.45
Sugar and sugar alcohols	Lactose	515.54
	Sorbitol	616.54
	Glucose	790.48

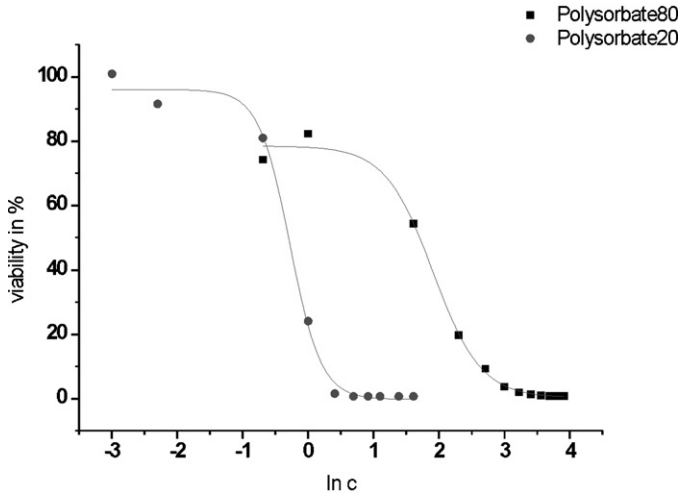

Fig. 5. Concentration dependent toxicity of polysorbate 20 and polysorbate 80 on Calu-3 cells having a different threshold (minimum concentration for toxic effect) and a different safety index (indicated by the slope of the curve).

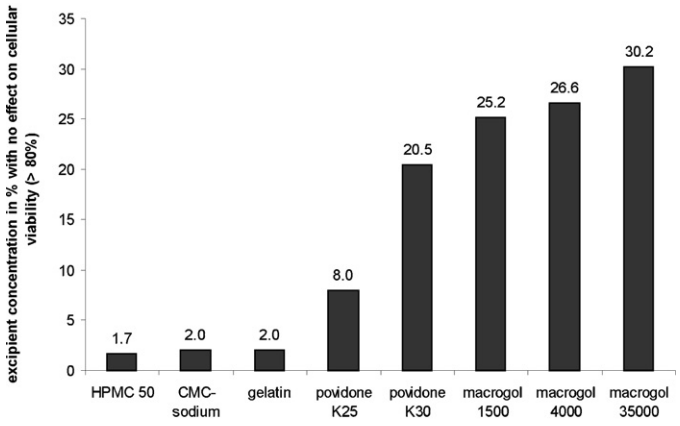

Fig. 6. Comparison of the concentration in % of polymeric substances being tolerated without a toxic effect on Calu-3 cells (calculated from viability data, $n = 4$).

formulations, where it is difficult to estimate the exact resulting concentration of a substance after application.

In the case of excipients used as solvents or co-solvents, a dependency of the toxic effect from the molecular weight was seen. This was true for the low molecular weight solvents as glycerol and propylene glycol having a higher LC_{50} value with lower molecular weight (2.8 M in the case of glycerol and 3.5 M for propylene glycol), but also for the homolog series of polyethylene glycols (macrogols), which were tested from a MW of 400 to 35,000 (Figs. 6 and 7). If the toxic effect of these excipients would mainly be due to osmolarity, similar LC_{50} values would be assumed for all substances. In contrast, highly hyper osmolar solutions of these substances were well tolerated as indicated by the MTT assay (Table 3).

Ethanol as a more lipophilic solvent exhibited a much higher toxicity with an LC_{50} of 771 mM than the other low MW solvents. This may be due to the greater ability of ethanol to dissolve components of the cellular membrane and with this causing cellular damage in comparison to the other tested solvents. Furthermore ethanol is known to denature proteins at higher concentrations and with this also exhibits a toxic effect. An evaluation of monolayer integrity with TEER showed that the epithelial resistance decreased considerably directly after application and recovery of the cellular barrier took longer with increasing ethanol concentrations (Fig. 8). Evaluation of viability after 4 h of incubation revealed

Table 3
pH and osmolarity of test substance solutions in HBSS + HEPES in the LC_{50} concentration ($n = 3$, ±standard deviation).

Substance	Concentration in HBSS + HEPES	pH	Osmolarity in mosmol
Macrogol 400	554 mM	6.6 ± 0.02	987 ± 6
Macrogol 1500	182 mM	7.2 ± 0.01	533 ± 6
Macrogol 4000	70 mM	7.2 ± 0.02	443 ± 21
Macrogol 35000	9 mM	7.2	370 ± 26
Glycerol	2.8 M	6.9 ± 0.01	3457 ± 12
Propylene glycol	3.5 M	7.1 ± 0.02	3967 ± 25
Lactose	515 mM	6.9 ± 0.01	863 ± 12
Sorbitol	617 mM	6.9 ± 0.02	1027 ± 46
Glucose	790 mM	6.9 ± 0.03	1207 ± 6
HBSS + HEPES	Pure	7.2 ± 0.02	348 ± 1

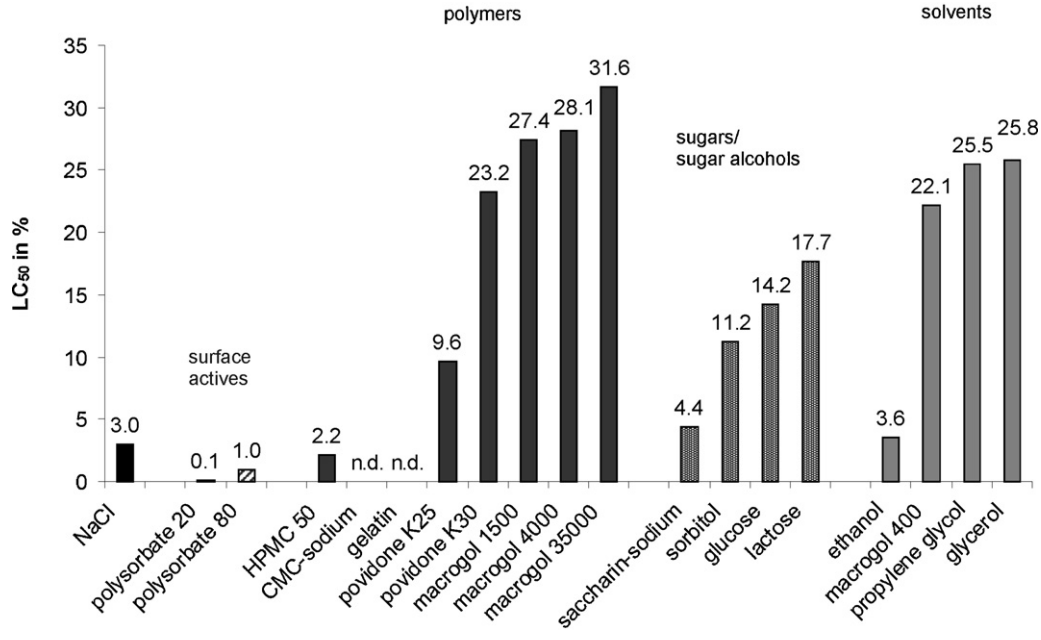

Fig. 7. Comparison of LC_{50} in % of the tested excipient groups (calculated from viability data, $n = 4$; n.d., not detectable due to high viscosity).

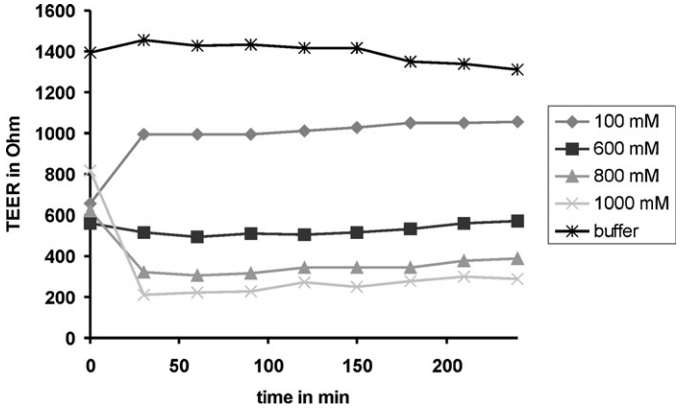

Fig. 8. TEER values of a Calu-3 monolayer cultivated on a membrane insert at different time points after application of different ethanol solutions (100 mM, 600 mM, 800 mM, 1000 mM and pure buffer); TEER prior to removal of medium and application of test solution 2212 Ω, resistance of filter membrane 162 Ω.

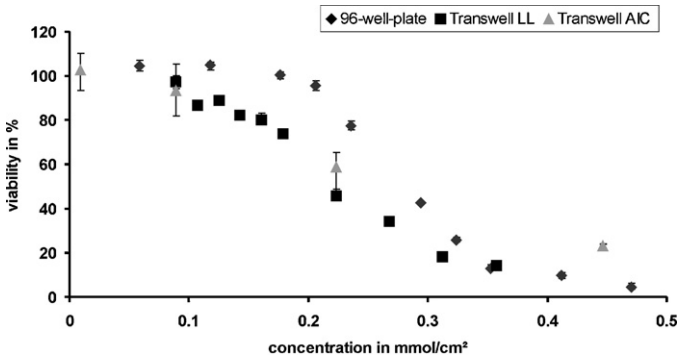

Fig. 9. Comparison of viability data after application of different concentrations of lactose obtained in different test systems (96-well plate with cell layer on the bottom, cells on membrane insert submersed under liquid and AIC), $n = 4$.

that the observed decrease in monolayer resistance correlated with the number of viable cells. The initial decrease in TEER can be judged as immediate reaction to the application of an irritant substance. Depending on the concentration, the cells are damaged more or less resulting either in cellular death or in recovery and with this in reconstruction of the monolayer integrity over time. With respect to formulation development, solvents are probably of minor interest, as most biomolecules do not tolerate solvents other than water. Further on, it would be generally advantageous to avoid solution formulations with respect to product stability.

The tested polymers perform differently depending on their substance characteristics. If the polymer is surface active as in the case of HPMC and carmellose-sodium, the toxic effect was more pronounced than from indifferent polymers like povidone or the solid polyethylene glycols (Fig. 7). Some polymeric excipients such as gelatin could not be tested in concentrations allowing an LC_{50} calculation as they result in viscous solutions not being applicable to the cell culture. Therefore, they could only be compared regarding the concentration without effect on cellular viability (Fig. 6).

Within the group of sugars and sugar alcohols, the monomer glucose exhibited the lowest toxicity with an LC_{50} of 790 mM, whereas the dimer lactose showed a higher toxicity exhibiting an LC_{50} of 515 mM (Table 2) and the sugar alcohol sorbitol had an intermediate toxic effect with an LC_{50} of 617 mM. If the LC_{50} in % was considered, this ranking was changed due to the different molecular weights, being almost similar for glucose and sorbitol, but doubled for lactose. This resulted in a different rating of the substances' toxicity with an LC_{50} of 11.2% for sorbitol, 14.2% for glucose and 17.7% for lactose (Fig. 7). However, the toxic effect was detected at far higher concentrations than in the group of surface active substances or polymers. The toxic effect could again not only be explained by osmolarity and must therefore be substance specific (Table 3).

In summary, different groups of excipients were tested due to their acute toxicity on the respiratory epithelial cell line Calu-3 utilising the presented MTT based assay. They differed in their toxic effects with the highest toxicity in the group of surface active substances, whereas solvents were much less toxic and the polymers exhibited varying LC_{50} values as summarised in Fig. 7. For data rating, the LC_{50} of lactose can be looked at as non-toxic excipient, as the toxicity of lactose has been well evaluated *in vivo* and the substance is regarded as being nontoxic in the respiratory system (Baldrick and Bamford, 1997). A drawback of the described toxicity assay is the limitation to buffer-soluble substances. This excludes both lipophilic excipients and in some cases also high concentrations of excipients due to an excess of maximum solubility (e.g. in the case of β-cyclodextrine) or a great increase in viscosity (especially for macromolecules) resulting in a semi-solid preparation not being capable of pipetting. To overcome these problems, the Calu-3 cell line was cultivated as Air-Interface-Culture (AIC), where the test substances could be applied directly to the cellular surface as powder without dissolving them. The results were comparable with cells grown submersed in the Transwell® system being a little more sensitive than cells cultivated submersed at the bottom of a 96-well plate (Fig. 9). Chitosan, a mucoadhesive excipient with uptake enhancement qualities, could only be assessed using an AIC, as the polymer was not soluble at pH ~7. The LC_{50} value of chitosan as assessed using this assay was 21.3 mg/cm^2, whereas lactose had an LC_{50} of 88.4 mg/cm^2 on the membrane insert culture. Hence, chitosan has to be judged as more toxic than lactose, but when the normally used amounts being lower in the case of chitosan are taken in consideration, the toxicity might be comparable.

Looking at the Calu-3 system itself, it has to be mentioned, that it is a monoculture of respiratory epithelial cells, which does not represent the complete respiratory epithelium. This could be realised by breeding a coculture system where macrophages and dendritic cells are present as well, with this more closely resembling the *in vivo* situation and possibly also behaving differently from the monoculture. Nevertheless, the here-presented model can be used as a simple model for *in vitro* comparisons.

In the literature, the difference between irritancy and toxicity has been well discriminated. Whereas irritancy might cause an aversion against a product due to a negative feeling (burning and itching), but does not necessarily result in cellular damage, toxicity may be impalpable, but results in remarkable cellular damage (Davies and Illum, 2003). So, for further assessment of formulations, the characterisation of possible irritant effects has to be kept in mind. The limitation of such a test is the predictability of what happens *in vivo*. As the *in vitro* system just represents a small part of the living system, these models often fail to show a good *in vitro–in vivo* correlation of the results (Sayes et al., 2007). However, there are promising results showing a good correlation (Kilgour et al., 2000). This may especially be true for acute effects, where a direct substance–cell interaction is looked at, whereas chronic effects remain difficult to mimic *in vitro*.

5. Conclusion

Both neutral red assay and MTT assay can be used for the evaluation of acute toxic effects of excipients on Calu-3 cells, with the MTT assay being more sensitive. Both tests are easy to perform and give reproducible results in the liquid culture as well as in the Air-Interface-Culture. The MTT assay is a useful and sensitive test for the determination of the acute toxicity of excipients in an *in vitro* system based on Calu-3 cells representing a good model for the res-

piratory epithelial cells and with this also serving as suitable model when evaluating acute toxic effects. The LDH assay is not suitable for the determination of acute toxic effects on Calu-3 cells. This is probably due to a direct interference of the test substances present in the medium with the enzyme. The LC_{50} is a good measure to evaluate the potential toxicity with respect to the concentration of excipient in the formulation or at the site of application, respectively. As novel excipients can be compared to well known and used excipients such as lactose or polysorbates, it will be possible to select excipients with a comparable low toxicity for further formulation development.

References

Agu, R.U., Ugwoke, M.I., Armand, M., Kinget, R., Verbeke, N., 2001. The lung as a route for systemic delivery of therapeutic proteins and peptides. Respir. Res. 2, 198–209.

Baldrick, P., 2000. Pharmaceutical excipient development: the need for preclinical guidance. Regul. Toxicol. Pharmacol. 32, 210–218.

Baldrick, P., Bamford, D.G., 1997. A toxicological review of lactose to support clinical administration by inhalation. Food Chem. Toxicol. 35, 719–733.

BéruBé, K., Aufderheide, M., Breheny, D., Clothier, R., Combes, R., Duffin, R., Forbes, B., Gaça, M., Gray, A., Hall, I., Kelly, M., Lethem, M., Liebsch, M., Merolla, L., Morin, J., Seagrave, J., Swartz, M., Tetley, T., Umachandran, M., 2009. In vitro models of inhalation toxicity and disease. The report of a FRAME workshop. Altern. Lab. Anim. 37, 89–141.

Davies, S.S., Illum, L., 2003. Absorption enhancers for nasal drug delivery. Clin. Pharmacokinet. 42, 1107–1128.

DeGeorge, J.J., Ahn, C.H., Andrews, P.A., Brower, M.E., Choi, Y.S., Chun, M.Y., Du, T., Lee-Ham, D.Y., McGuinn, W.D., Pei, L., Sancilio, L.F., Schmidt, W., Sheevers, H.V., Sun, C.J., Tripathi, S., Vogel, W.M., Whitehurst, V., Williams, S., Taylor, A.S., 1997. Considerations for toxicology studies of respiratory drug products. Regul. Toxicol. Pharmacol. 25, 189–193.

Fjellestad-Paulsen, A., Höglund, P., Lundin, S., Paulsen, O., 1993. Pharmacokinetics of 1-deamino-8-D-arginine vasopressin after various routes of administration in healthy volunteers. Clin. Endocrinol. (Oxf.), 177–182.

Foster, K.A., Avery, M.L., Yazdanian, M., Audus, K.L., 2000. Characterization of the Calu-3 cell line as a tool to screen pulmonary drug delivery. Int. J. Pharm. 208, 1–11.

Fotakis, G., Timbrell, J.A., 2006. In vitro cytotoxicity assays: comparison of LDH, neutral red. MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. Toxicol. Lett. 160, 171–177.

Grainger, C.I., Greenwell, L.L., Lockley, D.J., Martin, G.P., Forbes, B., 2006. Culture of Calu-3 cells at the air interface provides a representative model of the airway epithelial barrier. Pharm. Res. 23, 1482–1490.

Gonda, I., 2007. Peptides and proteins: pulmonary absorption. In: Encyclopedia of Pharmaceutical Technology ,. Informa Healthcare, Inc., USA, pp. 2731–2738.

Gupta, M., Gupta, A.K., 2004. In vitro cytotoxicity studies of hydrogel pullulan nanoparticles prepared by aot/n-hexane micellar system. J. Pharm. Pharm. Sci. 7, 38–46.

Hansen, M.B., Nielsen, S.E., Berg, K., 1989. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J. Immunol. Methods 119, 203–210.

Kaye, R.S., Purewal, T.S., Alpar, O.H., 2009. Development and testing of particulate formulations for the nasal delivery of antibodies. J. Control. Release 135, 127–135.

Kilgour, J.D., Simpson, S.A., Alexander, D.J., Reed, C.J., 2000. A rat nasal epithelial model for predicting upper respiratory tract toxicity: in vivo–in vitro correlations. Toxicology 145, 39–49.

Matilainen, L., Toropainen, M., Vihola, H., Hirvonen, J., Järvinen, T., Jarho, P., Järvinen, K., 2008. In vitro toxicity and permeation of cyclodextrins in Calu-3 cells. J. Control. Release 126, 10–16.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity tests. J. Immunol. Methods 65, 55–63.

Patton, J.S., 1996. Mechanisms of macromolecule absorption by the lungs. Adv. Drug Deliv. Rev. 19, 3–36.

Rote Liste, 2009. Rote Liste® Service GmbH, Frankfurt.

Rothen-Rutishauser, B., Blank, F., Mühlfeld, C., Gehr, P., 2008. In vitro models of the human epithelial airway barrier to study the toxic potential of particulate matter. Expert Opin. Drug Metab. Toxicol. 4, 1075–1089.

Sayes, C.M., Reed, K.L., Warheit, D.B., 2007. Assessing toxicity of fine and nanoparticles: comparing in vitro measurements to in vivo pulmonary toxicity profiles. Toxicol. Sci. 97, 163–180.

Steimer, A., Haltner, E., Lehr, C.-M., 2005. Cell culture models of the respiratory tract relevant to pulmonary drug delivery. J. Aerosol Med. 18, 137–182.

Steinberg, M., Borzelleca, J.F., Enters, E.K., Kinoshita, F.K., Loper, A., Mitchell, D.B., Tamulinas, C.B., Weiner, M.L., 1996. A new approach to the safety assessment of pharmaceutical excipients. Regul. Toxicol. Pharmacol. 24, 149–154.

von Wichert, G., von Wichert, P., 2006. Problems and chances of the application of systemically acting drugs via the lung. Med. Klin. 101, 718–723.

Weyermann, J., Lochmann, D., Zimmer, A., 2005. A practical note on the use of cytotoxicity assays. Int. J. Pharm. 288, 369–376.