Session 5.06
In vitro approaches for determining acute systemic toxicity

Estimating Acute Toxicity Based on *In Vitro* Cytotoxicity: Role of Biokinetic Modelling

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Summary
Deviations from a linear relationship between cytotoxic concentrations of a substance in vitro and toxic doses in vivo can result from the fact that effective concentrations in vitro are irrelevant for the concentrations that cause toxicity in target organs in vivo. An important reason is a compound's biokinetic behaviour, which determines the concentrations reached in target organs.
Physiologically-based biokinetic modelling will be employed to alert to chemicals displaying a deviating kinetic behaviour and that will thus need further attention to estimate their acute toxicity. This will improve the accuracy of estimating the in vivo toxic dose on the basis of in vitro basal cytotoxicity.

Keywords: acute toxicity, biokinetic modelling, cytotoxicity

Introduction

In the search for methods to evaluate the toxicological risk of chemicals without employing animal experimentation, much emphasis has been put on the replacement of acute toxicity (LD_{50}) determinations. One important assumption was that acute toxicity is related to a compound's basal cytotoxicity (Ekwall, 1983). In previous programmes, the feasibility of the use of *in vitro* cytotoxicity data for the prediction of *in vivo* lethal doses was tested, e.g. in the MEIC study (Ekwall et al., 1998), and by publication of the Register of Cytotoxicity (Spielmann et al., 1999; Halle., 2003). In these studies it was shown that basal cytotoxicity data resulted in good estimates for about 70% of the compounds, i.e. these chemicals could be classified in the appropriate LD_{50} classes (Clemedson and Ekwall, 1999). This implies that for about 30% of the cases the estimates on the basis of cytotoxicity data deviated from *in vivo* findings.

These deviations from a simple linear relationship between effective concentrations *in vitro* and toxic doses *in vivo* can result from the fact that the effective concentrations *in vitro* are irrelevant for the concentrations that may cause toxicity at the target site in target organs *in vivo*. This will in many cases be the result of the biokinetics of the compound under study (Blaauboer, 2002). For instance, the absorption of the compound may be minimal, thus leading to low systemic concentrations. Moreover, the processes of distribution, metabolism and elimination may lead to lower or higher concentrations in target organs than could be expected from an even distribution of the compound in the body. Thus, an important drawback of the use of cytotoxicity data is the difficulty of extrapolating a toxic concentration in the *in vitro* system to a toxic dose in the *in vivo* situation (Blaauboer, 2003).

The ACuteTox programme aims to improve the estimates for acute toxicity on the basis of non-animal studies (e.g. *in vitro* cytotoxicity studies). Thus, one of the areas needing attention is the study of the biokinetics of the test compounds. Two work packages in the programme are devoted to this: work package 5 involves studies of absorption, distribution and elimination, while work package 6 is devoted to the role of biotransforma-

tion. Other parts of the programme are devoted to other possible reasons for the absence of a clear relationship between basal cytotoxicity and *in vivo* acute toxicity, i.e. when specific organ toxicity is the most sensitive parameter for acute toxicity.

Biokinetic modelling

During the past decades the possibilities to estimate the concentrations of compounds in the different organs and tissues has been greatly improved by the application of simulation models describing these concentrations over time. These so-called physiologically-based biokinetic (PBBK) models (also described as pharmacokinetic models: PBPK) have proven to be very useful in many areas of toxicology and risk assessment (Andersen, 1991; 2003). In essence, these models enable the estimation of the concentration-time relationship of compounds, making use of two essential blocks of information: 1) the known anatomy and physiology of the organism; 2) the physico-chemical properties of the compound. The models describe the relevant anatomical structures such as liver or kidney, or tissue types such as fat or muscle. Tissue volumes and blood flow rates are important parameters in these descriptions. Distribution of a compound throughout the body is described by tissue–blood PCs and, if necessary, by any active transport or biotransformation processes. These species-specific and compound-specific parameters form part of a set of differential equations that describe the biokinetic behaviour of a compound. The feasibility of this modelling approach has been greatly increased by the availability of computer techniques that allow for the simultaneous, numerical solution of differential equations (Clewell and Andersen, 1986).

Using these models, it is possible to extrapolate from one exposure route to another by re-parameterising only the relevant uptake process, as the description of the distribution and elimination processes remains valid. Beside route-to-route extrapolation, the biologically based approach of PBBK models also allows extrapolation of dose and animal species beyond the conditions of laboratory studies (Andersen, 1991).

Biokinetic modelling based on *in vitro* and other non-animal data

The quality of biokinetic models heavily relies on the accuracy of the parameters used to build the models. Many models described in the literature are, at least for the major part, based on parameters measured in *in vivo* experiments. These experiences have resulted in the accumulation of data on parameters describing the physiology of organisms. Literature data now exist for a number of species, including laboratory rodents and humans. These data not only describe the "standard" rat or the "standard" human, but may also allow taking into account the known variability in these parameters, e.g. body weight. On this basis, a number of other parameters can be scaled as a function of body weight, e.g. organ volumes, blood flows, etc. Thus, it is possible to parameterise the models for their physiological parameters in a flexible way without doing experiments.

This is not so obvious for the compound-related parameters. Absorption via the oral, inhalatory or dermal route relies not only on factors determined by the anatomy and physiology of the organism, but also on the physico-chemical characteristics of the compound, and – most importantly – on the interplay between these characteristics and the physiology. The same applies to the distribution of the compound over the organs and tissues as well as to the elimination (excretion, biotransformation).

Attempts have been made to quantify these parameters on the basis of physico-chemical data. This has been done successfully for a number of these parameters, e.g. blood-air and blood-fat partitioning can be described well using quantitative property-property relationships (QPPRs) based on volatility and lipophilicity (Poulin and Krishnan, 1996a; Poulin and Krishnan, 1996b; DeJongh et al.,1997). Other processes are more difficult to describe. QPPRs have been developed for dermal absorption, but these relationships are often limited to certain chemical classes (Wilschut et al., 1995). The same applies for distribution to other tissues, such as the central nervous system, and also for excretion processes and for biotransformation. One important cause is the existence of transport and biotransformation processes that are not easily quantifiable on the basis of structure and physico-chemical properties alone. In these areas further experimental information is needed. The development and validation of *in vitro* methods in these areas is highly desirable (Coecke et al., 2005). In a number of these areas progress is being made, e.g. *in vitro* methods to determine oral absorption, making use of Caco-2 cell systems (Artursson and Borchardt, 1997), co-cultures to quantify blood-brain barrier transport (Prieto et al., 2004), hepatocyte cultures to measure biotransformation parameters (Treijtel et al., 2004).

Extrapolating *in vitro* toxic concentrations to *in vivo* toxic doses: integration of *in vitro* derived toxicodynamic data and biokinetic modelling

A number of studies have attempted to improve the predictive power of *in vitro* cytotoxic concentrations to estimate *in vivo* toxic doses, making use of biokinetic models for which the parameters were derived from *in vitro* and other non-animal data. In the ECITTS programme, the neurotoxicity of 10 chemicals was estimated on the basis of *in vitro* neurotoxic concentrations and biokinetic modelling. For the majority of these compounds, good predictions could be made for their lower-effect levels found in the literature (DeJongh et al., 1999; Blaauboer et al., 2000; Forsby and Blaauboer, 2003).

Another study applying this integration of *in vitro* cytotoxicity and biokinetic modelling was recently published by Gubbels-van Hal et al. (2005). In this study, six endpoints, among them acute toxicity for fish and for rodents, were estimated and compared with traditionally determined (*in vivo*) data from the same laboratory. This parallel testing gave the same acute toxicity classification for all compounds for fish and for 9 out of 10 compounds for rodents.

These studies form the "proof of concept" for the approach of integration of data derived from different domains in toxicolog-

ical hazard and risk assessment, as proposed earlier (Blaauboer et al., 1999; Health Council of the Netherlands, 2001).

The relevant *in vitro* toxic concentrations

One prerequisite for making useful extrapolations from *in vitro* toxic concentrations to *in vivo* toxic doses is that the proper concentrations are taken into account. *In vitro* systems consist of a biological component (i.e. the cell culture), a medium component (with or without serum or other proteins) and a physical component (the test tube or culture plate). Adding a certain amount of compound to the medium will result in a certain concentration to which the cells are exposed. This concentration is highly dependent on the characteristics of the different components in the system, as well as on the processes occurring at the borders of the components (e.g. binding to plastic, evaporation, etc.). Therefore, it is necessary to pay attention to these characteristics and processes. Taking the "nominal" concentration as a basis for further steps in the above-described integrative approach might lead to erroneous extrapolations. Sources of error are: protein binding (Vaes et al., 1997; Seibert et al., 2002; Heringa et al., 2004), the amount of medium in relation to the amount of cells, as well as the aforementioned binding to plastic and evaporation.

These considerations have led to the development of the concept of "*in vitro* biokinetics".

Improving the correlation between *in vitro* cytotoxicity data and acute toxic doses: alerts and correctors for biokinetic behaviour

The main objective of the ACuteTox programme is to produce a reliable system in which the acute toxicity of a compound can be estimated on the basis of non-animal data. The starting point is the determination of basal cytotoxicity. The finding that this parameter is not always a good predictor for acute toxicity *in vivo* stresses the need for the development of a logical and transparent strategy. In such a strategy decisions are made on the requirement of more data than basal cytotoxicity alone. In other words: when do we need to know more about a compound's biokinetic behaviour or its specific organ toxicity and how can we use this knowledge for the estimation of *in vivo* acute toxicity?

When confronted with a newly introduced compound of which the chemical and spatial structure is known, what are the means by which we can be alerted, without further experimentation than standard *in vitro* cytotoxicity testing, to it displaying potential for *in vivo* acute oral toxicity different to what might be expected from its *in vitro* cytotoxicity?

Questions to be answered are then:

- Can we expect that the compound will be absorbed efficiently via the relevant route of exposure? If a compound has a very low rate of absorption, this will lead to low internal exposure.
- Is it to be expected that the compound will have a distribution pattern *in vivo* that will lead to higher or lower concentrations near target cells? The answer to this question is important for the interpretation of basal cytotoxicity data, but can also be used to find clues for organ-specific toxicity.
- Is it to be expected that a compound would be metabolised to a compound with either a higher or with a lower toxicity?
- Can we expect that a compound will have a specific toxic effect that would cause acute toxicity? In that case, basal cytotoxicity would probably underestimate the *in vivo* acute toxicity.
- For which compounds will it be necessary to take *in vitro* biokinetics into account? This question refers to the relevance of a "nominal" concentration in the *in vitro* system vs. the "free" concentration.

From our earlier experiences, as described in the previous paragraphs, it is clear that tools to answer a number of these questions have been developed or are in the process of development. This enables further honing of a strategy. A first step in such a strategy would be to find those parameters in the structure of the compound or in its physico-chemical characteristics that can act as alerts for further action. A further step would include the use of knowledge on comparable or similar compounds (read across). Several papers on the relation between molecular properties and ADME-properties of compounds (QPPRs) (Poulin and Krishnan, 1996a; Poulin and Krishnan, 1996b; DeJongh et al., 1997) and even commercial software packages exist that give answers to at least one aspect of each of these questions.

Based on the estimated ADME-properties, a PBBK model can be developed and free plasma concentrations can be calculated. If one assumes that the *in vitro* culture medium free concentration represents this concentration, corresponding LD_{50} estimations follow. When a metabolite is the toxic agent, its DME properties should be estimated from its physicochemical properties and the corresponding PBBK model is developed.

In the realm of biokinetic studies, the emphasis in the ACuteTox programme will be put on the following aspects, since these are considered to be the most sensitive parameters:

- prediction of biokinetic behaviour on the basis of physico-chemical properties
- *in vitro* measurement of parameters relevant for oral absorption and development of QPPRs for these parameters
- *in vitro* measurement of parameters relevant for blood-brain barrier passage and development of QPPRs for these parameters
- *in vitro* measurement of the free concentration of compounds and development of predictors for deviating *in vitro* biokinetic behaviour
- PBBK modelling for those compounds that will be outliers in the simple relationship between *in vitro* LC_{50} and LD_{50} values.

These elements shall form the framework of an important part of a logical and transparent strategy for the estimation of acute toxicity values.

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ACuteTox – Optimation and Pre-validation of an *In Vitro* Test Strategy for Predicting Human Acute Toxicity

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Summary

ACuteTox is an integrated project under the EU-FP6 with the aim to develop a simple and robust in vitro testing strategy for prediction of human acute systemic toxicity, which could replace animal tests used for regulatory purposes.

Studies show good correlation of over 70% between in vitro basal cytotoxicity and rodent LD_{50} values or human lethal blood concentrations. However, a number of discrepancies occur which result in misclassification. ACuteTox aims to identify factors that can eliminate these misclassifications. The outliers in the in vitro/in vivo correlation will be evaluated in order to introduce further parameters (ADE, metabolism and organ specificity), which might improve the correlation. Integration of alerts and correctors in a prediction algorithm, together with implementation of medium throughput approaches, would allow establishment of a new testing strategy to better predict toxic classification.

Keywords: acute toxicity, kinetics, in vitro, in silico, testing strategy, target organ toxicity

Introduction

Validated alternative test methods are urgently required for toxicological safety testing of drugs, chemicals and cosmetics. Both REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) and the 7th amendment of the Cosmetics Directive (deadline for acute toxicity is 2009) call for the broad replacement of animal experiments on a short-term basis. Animal tests for topical toxicity have been successfully replaced one by one by alternative methods. In systemic toxicity, on the other hand, only some reduction or refinement methods have been developed. The aim of ACuteTox, which started on the 1st of January 2005, is to develop a simple and robust *in vitro* testing strategy to predict human acute systemic toxicity, which could replace the animal acute toxicity tests used today for regulatory purposes.

The extensive amount of work performed since the 70's produced a great number of *in vitro* models for acute systemic toxicity. Many studies have shown a relatively good correlation between *in vitro* basal cytotoxicity data and rodent LD_{50} values. In addition, the MEIC (Multicenter Evaluation of *In vitro* Cytotoxicity) programme showed a good correlation (around 70%) between *in vitro* basal cytotoxicity data and human lethal blood concentrations. However, this correlation means that a certain number of misclassifications have to be faced when using the existing tests. ACuteTox aims to improve this correlation to a level sufficient to ensure a valid prediction of acute toxicity.

ACuteTox is based on two major ongoing activities in this field, i.e. the ECVAM/ICCVAM validation study for basal cytotoxicity tests and the EDIT (Evaluation-guided Development of *In vitro* Test batteries) programme (the continuation of the MEIC study).

The MEIC and the EDIT programmes

The aim of the MEIC study, directed by Björn Ekwall, was to evaluate the relevance of using *in vitro* toxicity tests to predict human acute systemic toxicity (Clemedson et al., 1996a, 1996b, 1998a, 1998b and 2000; Ekwall et al., 1998a, 1998b and 2000).

In summary, an average EC_{50} of ten 24 h exposure tests with human cell lines predicted human peak concentrations from LC_{50} curves better (R^2=0.74) than the prediction of human lethal doses by LD_{50} for rats and mice (R^2=0.60-0.66) for 50 reference chemicals. When some known human toxicokinetic data (knowledge of the passage across the blood-brain barrier (BBB) and the timing of the lethal action) was used together with the cytotoxic concentration to predict human lethal concentrations, prediction increased considerably. The results demonstrated a high relevance of using human cell tests to predict human acute toxicity of chemicals, but showed also that other important toxic mechanisms exist, which may only be measured by supplementary *in vitro* toxicity tests, and that modelling of human toxicity was improved by additional toxicokinetic data, which can probably be obtained by new *in vitro* kinetic tests. These results encouraged Björn Ekwall to initiate the EDIT-project in 1998 (Ekwall et al., 1999). The main aim of the EDIT programme was

to optimise the original MEIC test battery by establishing and validating new *in vitro* tests relevant to biokinetics and tests for organ-specific toxicity.

The ECVAM-ICCVAM validation study of two *in vitro* basal cytotoxicity tests

During the International Workshop on *In vitro* Methods for Assessing Acute Systemic Toxicity (ICCVAM, 2001a), recommendations were made on the need to evaluate two sufficiently developed and standardised basal cytotoxicity assays for their ability to predict rodent LD_{50} values by using the RC (Register of Cytotoxicity) regression model in order to improve the dose selection for *in vivo* studies, and to predict human lethal concentration (ICCVAM, 2001b). In 2002 ECVAM and ICCVAM designed and started a joint validation study. 72 chemicals, which comprise the majority of the 50 MEIC chemicals, and chemicals nominated by different regulatory authorities, were selected according to the availability of acute oral rodent and human toxicity data. These chemicals have been tested in the BALB/c 3T3 cell line and in normal human keratinocytes using the neutral red uptake assay. A preliminary analysis of 12 chemicals showed a good correlation for both cell types between *in vitro* IC_{50} values and human peak concentrations from LC_{50} curves (Casati et al., 2005).

Methods

The starting point for ACuteTox is the existing correlation of *in vitro/in vivo* data (about 70%). Efforts will be dedicated to improve the correlation. This will be done by evaluating outliers of the *in vitro/in vivo* correlation in order to introduce further parameters, such as ADE (administration, distribution and elimination), metabolism and organ specificity, which might improve the correlation. Establishment of a new testing strategy with a better prediction of toxic classification will be possible by integration of alerts and correctors in a prediction algorithm, and by implementation of medium throughput approaches. "Alerts" refer to test/modelling indicating that the given chemical might deviate from the correlation and thus the prediction cannot be

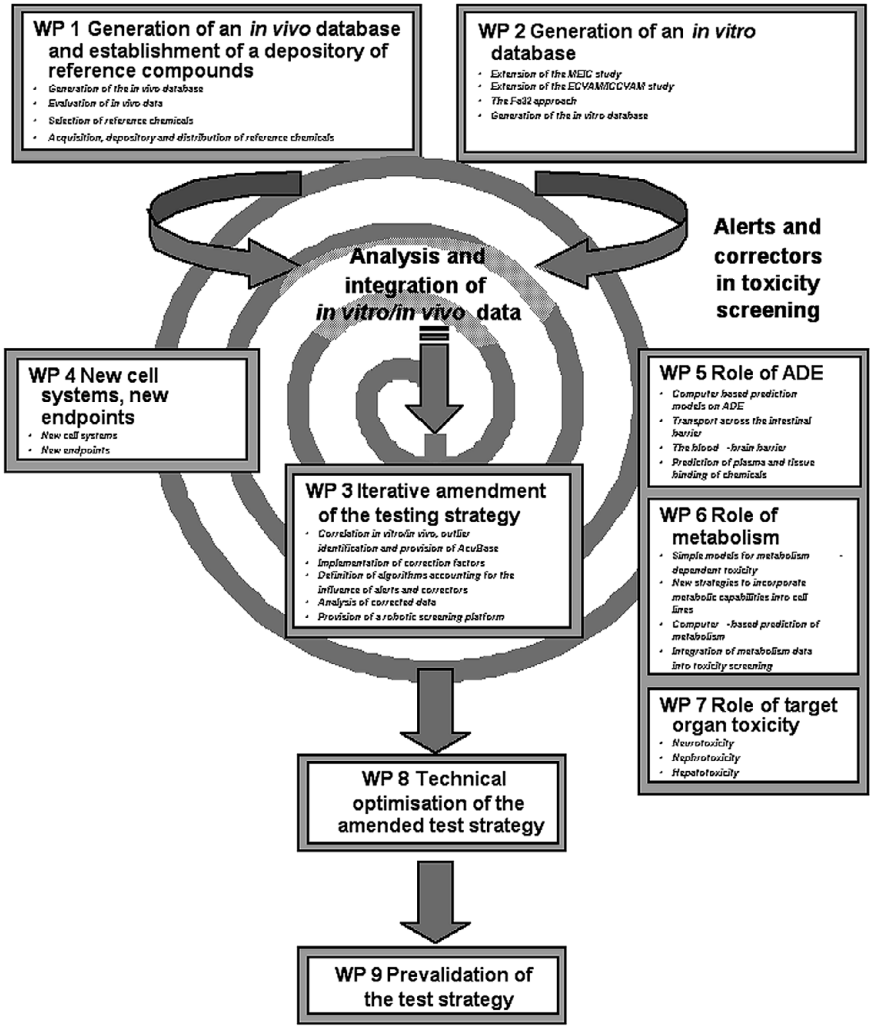

Fig. 1: The structure of the ACuteTox project.

trusted. "Correctors" refer to correction factors amending the result of the prediction.

The work within the project is divided into nine work packages (WPs). Figure 1 shows the outline of the project and the work in the different WPs is summarised below.

Work package 1: Generation of an *in vivo* database

The major objective of this WP is to generate a database containing high quality human and animal data. The *in vivo* databases obtained in the MEIC and ECVAM/ICCVAM studies, containing *in vivo* toxicity data for about 90 chemicals, will be merged to a new database. In total, data for 100-140 chemicals will be gathered. The data will be used in the evaluation of *in vitro* data obtained in WP2-7 and finally in the evaluation of the *in vitro* testing strategy. The animal tests used today in acute toxicity for regulatory purposes have not been properly validated. Therefore, some biometrical retrospective studies with the aim to evaluate the quality and predictive capabilities of the *in vivo* data will be performed. The outcome of these studies will give the reference of how accurate the ultimate *in vitro* test strategy has to be in order to be acceptable for regulatory purposes. Another aim of this WP is to select relevant reference chemicals for the pre-validation study in WP9.

Work package 2: Generation of an *in vitro* database

The aim of this WP is to merge cytotoxicity data from two ongoing projects (ECVAM/ICCVAM and MEIC/EDIT) and to increase the new database by extending the two studies. New testing will be performed in order to get a complete set of cytotoxicity data for the reference chemicals of the MEIC and ECVAM/ICCVAM studies. In addition, a reduced number of compounds showing specific organ toxicity (neuro-, nephro- and hepatotoxicants) have been selected and will be tested. The data will be generated in human primary keratinocytes, human (HepG2, HL-60), mouse (Balbc/3T3) and rat (Fa32) cell lines with different endpoints (protein content, ATP content, and neutral red uptake). The final outcome of WP2 will be a database containing high quality *in vitro* toxicity data obtained with defined Standard Operating Procedures.

Work package 3: Iterative amendment of the testing strategy

The aim of this WP is to reduce step by step the outliers in the *in vitro/in vivo* correlations (data from WP1 and WP2) by examining the underlying reasons for deviation such as the quality of data and lack of ADME or target organ specificities. For this purpose, the WP will evaluate new cell systems and/or endpoints (WP4), and introduce, in an iterative manner, corrector and alert assays for ADE, metabolism and target organ toxicity (WP5-7). This requires detailed characterisation of the outliers that are generated during the process. This iterative approach will allow, in a sequential manner, improvements of the prediction for acute toxicity. During the iterative process, the assays that are identified as THE candidates for corrector and alert assays, based on the improved ability to model toxic and lethal doses, will be adapted and transferred to a robotic system.

Work package 4: New cell systems and new endpoints

The aim of this WP is to provide an alternative way to improve the predictivity of cell-based cytotoxicity assays by incorporating more specific end-point parameters, and/or more appropriate cell systems. Cord blood cells and subpopulations thereof enriched in stem cells and human cell lines will be used as novel *in vitro* models for haematopoietic toxicity assays; colony forming unit-granulocyte/macrophage (CFU-GM), CFU-megakarocyte, and whole blood cytokine production assays. The feasibility of cytomic analysis adapted to robotic screening will be explored by the use of the newest developments and strategies to investigate cytotoxicity changes, by flow cytometric and biochemical assays. Also, endpoints such as oxidative stress and delayed toxicity will be evaluated in different cell systems.

Work package 5: Alerts and correctors in toxicity screening (I): Role of ADE

In this WP, the most crucial parts of the kinetic behaviour will be studied. This will be done, either by experimentally determining kinetic parameters by *in vitro* methods, or by computer-based kinetic modelling. The most crucial parts are: absorption of compounds, distribution between blood and tissues and the passage of special barriers. In the context of acute toxicity, the BBB is the most relevant special barrier and will receive extra attention. The value of *in vitro* determinations of toxic effects will further increase when kinetic parameters are taken into consideration. This will be done by modelling the *in vivo* kinetic behaviour of a compound, making use of physiologically-based biokinetic models (PB-BK).

Work package 6: Alerts and correctors in toxicity screening (II): Role of metabolism

To determine whether toxicity is associated with the metabolism of a compound, cytotoxicity in primary rat hepatocytes (metabolically competent cell) versus HepG2 (metabolically non-competent cell line) will be examined. By comparing the concentration-toxicity curves (or IC_{50} data) of the compound in both models it should be possible to ascertain whether the molecule elicits toxicity after or irrespective of its metabolism, indicating if bioactivation of the xenobiotic is required to elicit toxicity. A subset of reference chemicals, containing compounds for which metabolism is known to be involved in the toxicity mechanism and compounds for which toxicity is independent of metabolism, will be tested. Strategies based on engineered cells, including expression vectors for transient and controllable expression of biotransformation enzymes for CYP 1A2, 2A6, 2C9, 2E1 and 3A4, could be a way to overcome their intrinsic limitations by generation of metabolically competent cell lines. For this purpose, recombinant-defective adenoviral vectors encoding major CYP genes involved in foreign compound metabolism will be generated and used in HepG2 cells.

Work package 7: Alerts and correctors in toxicity screening (III): Role of target organ toxicity

The aim of WP7 is to explore the role of specific organ toxicity (liver, kidney and nervous system) in responses induced by out-

liers in the *in vitro/in vivo* correlation. Since the scientific expertise for each of the organs is very different, this WP is organised into three sub-WPs.

WP 7.1 Neurotoxicity

Lack of correlation between *in vivo* and *in vitro* data can arise from specific effects on main organs, which results in death of the living being without general cytotoxic effects. The vital functions of the central (CNS) and the peripheral nervous system (PNS) suggest these organs as candidates to explain the presence of outliers. Studies of the specific and general targets *in vitro* require well-characterised and complementary model systems. This WP therefore engages organotypic slice cultures, aggregating brain cell cultures, primary cell cultures and neuronal cell lines (also models for the PNS) to assess the effectiveness of *in vitro* models for acute neurotoxicity of a set of selected reference chemicals, including both specifically neurotoxic and general acutely toxic substances.

The major molecular mechanisms involved in stimulatory and inhibitory/depressive activity of the CNS and PNS will be explored. The following endpoints will be studied: $GABA_A$ receptor function, voltage-gated calcium channel function, GABA uptake, acetylcholine esterase-, cholineacetyl transferase-, glutamate decarboxylase-, glutamine synthase- and 2',3'-cyclic nucleotide phosphohydrolase activity, glial and neuronal differentiation, dendrite and axonal structures, Ca^{2+} homeostasis, total ATP content, mitochondrial membrane potential (MMP) and cell membrane potential (CMP), and production of ROS (reactive oxygen species). Effects on the global electric activity and glycolytic activity will be determined in complex brain cell aggregates. Investigations of new neurotoxicological target genes by gene array analysis will also be enrolled. The general cytotoxicity, measured as cell membrane disruption and cell death will be determined in each cell/organotypic model as a reference for the neurospecific effects.

WP 7.2 Nephrotoxicity

It is important to know whether the outliers in the existing correlation between *in vitro* and *in vivo* data present specific nephrotoxic effects. The kidney is especially susceptible to toxicity because of its role in excreting compounds, which involves a high blood supply, concentrating substances, metabolising substances and transporting substances. A major focus of this sub-WP will be the further development of *in vitro* models by analysis of a subset of nephro- and non-nephrotoxic compounds by means of trans-epithelial, paracellular permeability in the LLC-PK1 and MDCK renal tubular cell lines.

WP 7.3 Hepatotoxicity

This sub-WP is aimed at identifying new biomarkers for hepatotoxicity amenable to high throughput testing and capable of alerting to compounds that might show preferential or selective toxicity to the liver that would be underestimated by basal cytotoxicity tests. Efforts will be addressed to (1) design a screening strategy for hepatotoxicity amenable to robotic screening; (2) detection of new markers of hepatotoxicity and (3) design *in vitro* models to assess impairment of bile acid and bilirubin transport.

Work package 8: Technical optimisation of the amended test strategy

After data have been collected in the other WPs, full use of these data will be made by integrating the collected knowledge. This will be done using an integrated scheme in which the collected data are interpreted, and decisions are made.

The data incorporated into the integrated scheme will include:

- Physico-chemical properties of compounds (e.g. lipophilicity, reactivity, molecular weight, volatility, etc.), as well as the presence of certain structural alerts for specific types of biological activity. Where available and where possible, this part of the work will also include the application of quantitative structure activity relationships (QSARs).
- *In vitro* toxicity data, primarily basal cytotoxicity. Furthermore, data on selective cytotoxic reactions (i.e. specific disturbances of physiologically relevant processes in differentiated cell types) will be used to make estimates of concentrations leading to toxicity, which are relevant to acute toxicity *in vivo*.
- Kinetic data: estimates of data for absorption, distribution and elimination as well as metabolism. These data are crucial for the interpretation of all *in vitro* toxicity data and will have to be used to relate the relevant concentrations at which toxicity occurs in *in vitro* systems with a dose that will lead to such toxic levels in an intact organism.

The result of this WP will be an estimate of the toxic dose (or the toxic blood concentration) for the chemical under study. These data will be compared with known *in vivo* toxic dose levels from WP1. The integration of all available data will result in better predictions of toxicity. On the basis of the findings, a further developed testing strategy will be described, depicting the minimal requirements for testing with the highest possible accuracy.

Work package 9: Prevalidation of the test strategy

During the last 2 years of the 5-year project the testing strategy will be pre-validated. A detailed assessment of the reproducibility and relevance of each building block composing the strategy will be performed. The protocols will be amended to be sufficiently standardised and optimised to enter a formal validation process. Pre-validation of the most promising models will be carried out according to ECVAM's criteria by 3-4 laboratories. The results will be independently assessed and, if possible, recommendations for the regulatory acceptance of the developed testing strategy will be presented.

Discussion

In summary, ACuteTox aims to improve the prediction of acute toxicity using *in vitro* methods and, at the same time, to signal which compounds require further testing because their acute toxicity cannot be properly predicted. A pre-validated testing strategy and a pre-validated associated prediction model for acute systemic toxicity will be provided. It is expected that further validation will lead to regulatory acceptance and its incorporation into the set of standardised test guidelines for hazard

assessment of chemicals. The proposed testing strategy has the potential to replace EU methods B.1bis and B.1tris in Annex V of Dir 67/548 EEC and consequently the corresponding OECD Test Guidelines 420 and 423, as well as TG 425. This, in addition, will decrease the need for animal testing and will increase the harmonisation of testing protocols, both on EU level and on the global OECD level.

The implementation of REACH will result in the need for a further assessment of up to 30,000 existing chemicals, which are currently marketed in volumes greater than 1 ton p.a. It is estimated that the testing of these existing chemicals will result in the use of around 4 million animals. The costs for safety testing of chemicals are high and will increase tremendously when REACH is implemented in the EU. The optimisation of the *in vitro* testing strategy within the ACuteTox project will contribute to the establishment of less expensive and more scientifically-based safety testing. In addition, it will guarantee the predictivity of the developed testing strategy and will also provide a testing strategy that can be adapted to a robotic system, which, in the end, can deal with the requirements of the new chemicals policy in terms of testing high numbers of chemicals.

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Cell Culture Models of the Air-Blood Barrier for the Evaluation of Aerosol Medicines

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Summary:
Cell culture models of pulmonary epithelia offer excellent opportunities to study transport processes of drugs and other xenobiotics across the air-blood barrier, as well as to assess the inhalation safety of new polymers and other chemicals. After adequate characterisation and validation, such systems may be valuable alternatives to inhalation experiments with animals.
We have been evaluating the pulmonary epithelial cell lines Calu-3, 16HBE14o-, CFBE41o- and A549, as well as primary cultures of human alveolar epithelial cells (hAEpC). Typically, cells are grown on permeable filter supports, allowing the formation of monolayers with functional tight junctions and pharmaceutically relevant transporter proteins. While the cell lines Calu-3 and 16HBE14o- appear useful to model the bronchial epithelium, the cell line A549 develops only weak barrier properties. Therefore, it still appears necessary to use primary cultured cells to model the alveolar epithelium.

Keywords: epithelial permeability, cytotoxicity, pulmonary drug delivery, in vitro models

Introduction

The pulmonary route is of increasing interest for the development of new medicines, not only for the treatment of lung diseases (e.g. asthma, COPD) but also for the fast and efficient delivery of drugs into the systemic blood circulation. Advanced drug carriers, such as nanoparticles or liposomes, however, require the use of polymers and other excipients, the effects of which on the airway and respiratory epithelia are still relatively unknown, especially with regard to their safety.

While the safety and efficacy of new drugs and delivery technologies can only be judged by clinical studies in man, such tests are usually preceded by preclinical tests on animals. However, animals by their nature are rather complex systems and do not easily allow the acquisition of detailed information on the mechanisms of drug absorption at a given biological barrier, such as the air-blood barrier of the lung. Therefore, *in vitro* models of biological barriers are extremely useful, because they allow the study of biological processes at such barriers under controlled conditions. Apart from the possibility to use such systems for absorption/safety screening among larger numbers of candidates, studies at the cellular level provide a better understanding of critical factors and therefore allow optimisation of formulations. In the drug development process, where the first aim is to bring a new medicine into clinical testing as quickly as possible, such cell culture based *in vitro* test systems may significantly reduce the use of laboratory animals. In addition, candidate drugs, which have successfully passed the stage of *in vitro* testing, may be expected to have a better success rate in passing the next stages of more complex *in vivo* tests (s. fig.1).

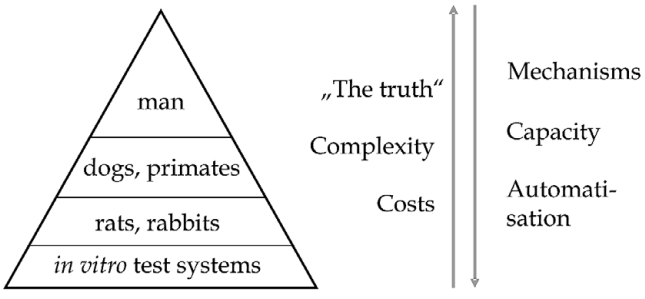

Fig. 1: *In vitro* test systems – the golden mean between manageability and explanatory power.

Functional anatomy of the lung regarding drug absorption and delivery

Some anatomical peculiarities of the human lung have led to an increasing interest in the pulmonary route for both local and systemic drug delivery. The large pulmonary epithelial surface estimated at 100-145 m^2 has almost the same resorption area of the intestinal mucosa, but only a small number of medicines are currently available for inhalation. Also, the high blood perfusion rate and the very small alveolar fluid volume of 7-20 ml are in favour of the lung as a novel route for the application of medicines. The alveolar epithelium in the deep lung is one of the thinnest barriers in the human body. The distance between the airspace and the capillary blood is in the range of 2 and 10 µm and can supposedly also be passed by relatively large molecules. However, not only the alveolar region should be contemplated but also the bronchial region. As conducting airways the bronchial tubes play a fundamental role in drug efficacy, especially for locally acting drugs to treat respiratory diseases such as asthma or COPD. In both the deep lung and

in the bronchial region the epithelium is the key for successful drug delivery.

Transepithelial transport processes
The function of an epithelium is the control of resorption or secretion of substances. Some substances like water can diffuse through the epithelium by using the intercellular space, but most substances need an energy dependent transport. Five main transport pathways are distinct (s. fig. 2): 1) paracellular diffusion; 2) transcellular diffusion; 3) carrier-mediated uptake at the apical domain followed by passive diffusion across the basolateral membrane; 4) transcytosis; 5) active secretion or efflux.

The airway epithelium of the bronchial region
According to the function of the airways – conduction, heating, clearance, and moisturisation of the air – the bronchial region has a specialised epithelium. It consists of columnar shaped cells. In the uppermost areas of the respiratory tract (nose, trachea, bronchi) it is multilayered, but tends to be thinner and monolayered towards the deeper (peripheral) lung. Mucus and ciliary transport are very effective clearance mechanisms. Airway goblet cells and submucosal glands form the major sources of human respiratory mucus. In the adult, mucus-secreting glands occupy about one-third of the inner airway wall wherever there is supportive cartilage.

The respiratory epithelium of the alveolar region
According to its major function, i.e. gas exchange, the alveolar epithelium is very thin (typically less than 1 µm). The largest part (70-90%) of its big surface area (100-140 m^2) is formed by so called alveolar epithelium type I (ATI) cells. They are spread-out in shape and form a tight squamous monolayered epithelium. Interspersed between the ATI cells are the alveolar epithelium type II (ATII) cells. They are relatively small, cuboidal in shape and more numerous than the ATI cells. They occupy only 10-30% of the surface area and are considered to be the progenitors of the ATI cells, because ATII cells may transform into ATI cells if some of the latter die or are damaged. However, the major function of the ATII cells is the production and secretion of lung surfactant.

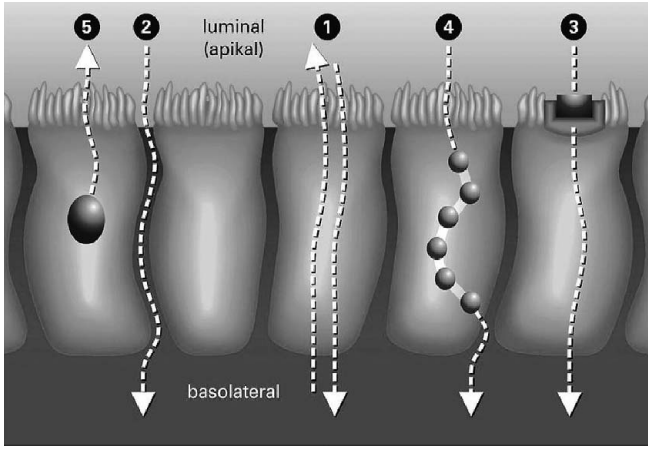

Fig. 2: Transport mechanism of drugs across an epithelium. 1. transcellular diffusion; 2. paracellular diffusion; 3. carrier-mediated uptake at the apical domain followed by passive diffusion across the basolateral membrane; 4. transcytosis; 5. active secretion or efflux. *Deutsche Apotheker Zeitung, 22.01.2004*, 61.

Cell culture models of pulmonary epithelia

Calu-3
Calu-3 is an adenocarcinoma cell line derived from a 25-year old Caucasian male. It has been suggested to express tight barrier properties on the basis of electrophysiological studies. The presence of tight junctional proteins was confirmed by immunoblotting, and functional properties of the monolayers were studied by measurement of transepithelial electrical resistance and mannitol permeability (Wan et al., 2000). Calu-3 cells have been the subject of relatively many investigations. After a few investigations on the equipment of the cells with ion channels or receptors, the cell line was used relatively soon as a tool for transport studies.

Mathia et al. (2002) studied the permeability characteristics of Calu-3 to passively and actively transported drugs and correlated the data with other *in vitro* models and with rat lung absorption *in vivo*. Air-interface cultured (AIC) Calu-3 cells grown on collagen-coated permeable filter supports formed "tight" polarised and well differentiated cell monolayers with apical microvilli and tight-junctional complexes. Solute permeability was dependent on lipophilicity, and inversely related to molecular size. Calu-3 cells actively transported amino acids, nucleosides and dipeptide analogues, but not organic anions, organic cations or efflux pump substrates. The permeability characteristics of Calu-3 cells correlated well with primary cultured rabbit tracheal epithelial cells *in vitro*, and the rate of drug absorption from the rat lung *in vivo*.

Apart from their use as a transport model, Calu-3 cells can also be employed for the investigation of metabolic processes. Borchard et al. (2002) cultivated Calu-3 cells on microporous filters at an air interface for 16-18 days, and incubated the cells with the glucocorticosteroid budesonide. With the aid of mass spectrometry of cell extracts, fatty acid conjugates of budesonide were detected. It seems that Calu-3 cells are able to store budesonide by intracellular conjugation. Therefore, it was suggested to use the Calu-3 cell model as a tool for examination of local pharmacokinetics and metabolism of glucocorticosteroids at the bronchial epithelium. Glucocorticosteroids were also employed for a study on the efflux system P-glycoprotein in Calu-3 cells (Hamilton et al., 2001). The P-gp modulation efficacy of glucocorticosteroids was determined by its ability to increase the accumulation of the P-gp substrate rhodamine 123 in the cells. Because of the high tightness and the easy cultivation conditions, Calu-3 cells are widely used for transport studies. Although Calu-3 is a bronchial (i.e. not alveolar!) epithelial cell line, it is often used also as a model of the pulmonary epithelium in general.

16HBE14o-

Another human bronchial epithelial cell line, 16HBE14o-, immortalised by virus transformation, also shows significant transepithelial resistance and can be used for transport studies. In comparison to the Calu-3 cells, the 16HBE14o- cell line seems to express more P-glycoprotein, lung resistance-related protein (LRP), and caveolin-1. Immunocytochemical staining showed expression of P-gp localised at the apical membrane of 16HBE14o- cell layers. The flux of rhodamine 123 across cell layers exhibited a greater apparent permeability (P_{app}) value for the secretory direction. This asymmetry disappeared in the presence of verapamil, a P-gp inhibitor. The 16HBE14o- cell line may be a suitable candidate for an *in vitro* model for mechanistic studies of drug transport processes involved in the smaller airways, because it shows drug transport systems that are also present in the human bronchus *in vivo* (Ehrhardt et al., 2003). To increase the simulation of the *in vivo* conditions, the cultivation of the cell lines under air-interface culture conditions was attempted. ZO-1, as indicator for the tight junctions, was found in cells grown in both AIC and LCC (liquid culture conditions). However, only LCC-grown cells exhibit protein ZO-1 localised as a zonula-occludens-like regular belt connecting neighbouring cells. The presence of typical tight junctions has been confirmed by electron microscopy. Immunostaining for occludin, claudin-1, connexin 43, and E-cadherin has demonstrated intercellular junction structures only in the cells in LCC. These morphological findings were paralleled by higher transepithelial electrical resistance values and similar fluxes of the hydrophilic permeability marker fluorescein-Na under LCC compared with AIC conditions (Ehrhardt et al., 2002). Because of the equipment of the cell with drug transport systems like P-gp or LRP, this cell line can be used for the investigation of the underlying transport pathways.

Also it has been shown that diesel exhaust particles can be phagocytosed by 16HBE14o- cells, inducing the release of cytokines (Marano et al., 2002).

CFBE41o-

The CFBE41o- cell line was generated by transformation of cystic fibrosis tracheo-bronchial cells with SV40. It is homozygous for ΔF508-CFTR over multiple passages in culture and expresses a number of proteins relevant in the context of pulmonary drug absorption, for example P-gp, LRP and caveolin-1 (Ehrhardt et al., 2005). Cystic fibrosis (CF) is a lethal genetic disease caused by a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR), which mainly functions as a chloride channel. The main clinical symptoms are chronic obstructive lung disease with excessive inflammation and chronic infection, which is responsible for most of the morbidity and mortality associated with CF, and pancreatic insufficiency. The CFBE41o- cell line should be useful for studies in the scope of CF gene transfer or alternative treatment using small drug molecules and gathering further knowledge about the disease on the cellular level, without the need for primary culture.

A549

The A549 cell line possesses alveolar epithelium type II cell phenotype and has been widely used as a system to study the regulation of pulmonary surfactant synthesis. However, cultured A549 cells do not undergo transition to form a phenotype similar to that of an ATI cell. Furthermore, although the A549 cell has received some attention as a monolayer culture for the study of solute transport, its cell architecture and barrier properties are quite distinct from that of an ATI cell monolayer. Thus, an *in vitro* cell model of the human alveolar epithelium possessing the relevant qualities of the alveolar epithelium *in situ* is definitely needed. The A549 line is a human lung adenocarcinoma derived by explant culture from the peripheral airways of a Caucasian male with lung cancer. A549 cells show a very high mannitol permeability coefficient, and approach the characteristics of cell-free filters. The "leaky" monolayers formed by these airway carcinoma cell lines failed to show significant immunostaining for the tight junction protein ZO-1 (s. fig. 3). The leaky formation of tight junctions in A549 is also the cause of very low transepithelial electrical resistance by their monolayers. This suggests that the formation of peripheral rings of ZO-1 staining is related to the formation of tight junctions and that these junctions probably determine low permeability to mannitol. A549 exhibited staining for desmoplakin, but no staining for E-cadherin. The functional tight junction deficits of the A549 cell line seem to preclude its use in permeability studies. Nevertheless, some authors have also reported comparatively high TEER (transepithelial electrical resistance) values and permeability rates for filter-grown A549 cells, which might be a question of optimised culture conditions

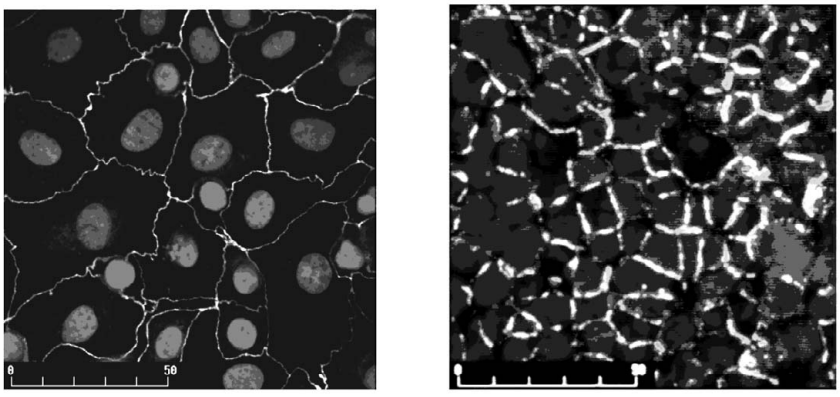

Fig. 3: Visualisation of tight junctional protein ZO-1 by specific antibody; nuclei counterstained by propidium iodide and observed by confocal laser scanning microscopy.

On the left, human alveolar epithelial cells in primary culture on day 8, and on the right A549 cells after 8 days in culture (Elbert et al.,1999).

(Rothen-Rutishauser et al., 2005). The use of immortalised cell lines is limited by the fact that in many cases the cells loose their characteristics during *in vitro* culture and will senesce after a certain number of cell divisions. Immortal cell lines from primary cultures are not a perfect representation of the original cells in primary culture. Because of these problems a great majority of researchers resort to the use of primary non-cancer cell lines.

Human Alveolar Epithelial cells in primary Culture (hAEpC)
Lung alveolar epithelium *in vivo* is composed of two specialised epithelial cell types, the squamous alveolar epithelial type I (ATI) cell and the surfactant-producing cuboidal alveolar epithelial type II (ATII) cell. Current evidence supports the hypothesis that ATII cells serve as the sole progenitors of ATI cells *in vivo* (Uhal, 1997; Fehrenbach, 2001). Accordingly, isolated ATII cells in culture lose their characteristic phenotype and acquire morphological and biochemical markers characteristic of ATI cells over a 5- to 10-day period. Morphological changes during differentiation include the generation of monolayers with high transepithelial electrical resistance (>1,000 Ohm * cm^2) and a loss of microvilli, an increase in the cell surface area and the development of thin cytoplasmic attenuations extending away from a protruding nucleus. The isolation of ATII cells predominantly from rat and rabbit lung tissue, and their culture over time leading to a primary culture of ATI-like cells is now an established technique for different purposes. Although the isolation of primary human alveolar cells has been described before (Alcorn et al., 1997), primary human cells are not commonly used as an *in vitro* model for the air-blood barrier. The isolation of human alveolar type II epithelial cells (hAEpC) and their primary culture, which results in confluent monolayers capable of generating tight junctional complexes and high transepithelial electrical resistance, was described by Elbert et al. (1999). The morphological cell change from an ATII phenotype to an ATI-like cell phenotype over culture time was described by Fuchs et al. (2003). Moreover, the formation of characteristic plasma membrane structures termed caveolae and the synthesis of their major structural protein, caveolin-1, was observed in these cells. The caveolae membrane system is of interest because of its potentially important role in macromolecule transport across the "air-blood-barrier" of the lung (Gumbleton et al., 2000), including both the clearance of endogenous protein from the airspace and the absorption of inhaled therapeutic protein. Primary type II alveolar cells (ATII) are isolated from human non-tumour lung tissue, which is obtained from patients undergoing lung resection. The isolation is performed according to a protocol described by Elbert et al. (1999). The isolated ATII cells are seeded on collagen/ fibronectin-coated polyester filter inserts using small airway growth medium containing penicillin and streptomycin and with the addition of low concentrations of foetal calf serum in order to suppress fibroblast growth. Formation of functional tight junctional complexes and generation of confluent monolayers is routinely determined by measuring TEER using an electronic voltmeter. After reaching confluence, the alveolar monolayers of hAEpC typically reveal TEER values of 1,000-2,000 Ohm * cm^2 on days 6-8 post seeding. The formation of tight junctions can also be routinely monitored by immunofluorescent staining for ZO-1 (Fuchs et al., 2003).

Pulmonary cell culture models in drug delivery research

Simple test systems are needed for drug absorption studies, especially in the early or exploratory phase of drug development. Excised lung tissue, isolated single cells, artificial membranes and *in vivo* models have distinct limitations. Therefore, the use of filter-grown tight cultures of pulmonary epithelial cells appears to be the most attractive alternative. While the use of similar models, such as e.g. the Caco-2 line, has already found much acceptance with respect to candidate screening for oral drug delivery, the development of comparable models with respect to pulmonary drug delivery is still in its beginnings. Their usefulness with respect to a better understanding of the transport mechanisms (which may also have impact on the design of advanced drug delivery systems) at the air-blood barrier has already been well recognised in the literature. However any prediction of *in vivo* pulmonary drug absorption based on such *in vitro* data can at present only be made most cautiously. In view of the complexity of pulmonary drug deposition (besides absorption!) and its poor control in many clinical studies, there is not enough data available yet to judge or postulate a reliable *in vitro/in vivo* correlation for such pulmonary cell culture systems at the present time.

With this disclaimer in mind, epithelial cell culture models of the lung still bear several advantages over other experimental techniques: (a) they are less time-consuming; (b) they enable rapid evaluation of methods for improving drug absorption; (c) they allow use of human rather than animal tissues; (d) they can help to reduce the number of animal studies.

Absorption and transport studies
Plating tight pulmonary cells on membrane supports, such as Transwell® cell culture inserts, allows the study of apical to basolateral (A → B) and basolateral to apical (B → A) drug transport. Membrane filters have been used as cell growth substrates since the 1950s.

For epithelial and other cell types, the use of permeable supports *in vitro* allows cells to be grown and studied in a polarised state under more natural conditions. Cellular functions, such as transport, adsorption, and secretion can also be studied, since cells grown on permeable supports provide convenient, independent access to apical and basolateral plasma membrane domains. Through taking samples from the receiver compartment, drug transport can be measured. The permeability of the investigated compound can be calculated by

$$P_{app} = (dQ/ dt)/ (A* C_0),$$

where dQ/ dt (mol s^{-1}) is the transport rate and indicative of the increase in the concentration of drug in the receiver chamber per

time interval. A (cm^2) is the surface area of the cell culture support, and C_0 (mol/ml) the initial drug concentration in the donor chamber.

Safety assessment/cytotoxicity
Inhalable nanoparticles are able to enhance drug or DNA stability for purposes of optimised deposition to the targeted lung areas. Surface modifications can mediate drug targeting. The suitability and non-toxicity of nanoparticles and the raw material of the nanoparticles have to be investigated *in vitro* on primary airway epithelium cells and cell lines like 16HBE14o- or Calu-3. The uptake of nanoparticles into these cells can be examined by confocal laser scanning microscopy or flow cytometry. The cytotoxicity of particles can be evaluated by a LDH (lactate dehydrogenase) release test and the inflammatory potential of the particles can be assessed by measuring interleukin (IL-8) release. Brzoska et al. (2004) for example reported with the aid of a 16HBE14o- model that protein-based nanoparticles are suitable drug and gene carriers for pulmonary application.

Cell compatible deposition systems of metered dose pharmaceutical aerosols
While toxicological studies on aerosols are typically focusing on the exposure to a xenobiotic at a certain concentration and over a given period of time, pharmaceutical aerosols are usually administered as a metered single dose. In order to estimate the bioavailability of a deposited dose, or to judge the biocompatibility of a once or repeatedly administered aerosol formulation, appropriate deposition chambers are needed which are able to address pharmaceutically relevant questions but at the same time are compatible with the implementation of the cell culture systems to study drug absorption subsequent to deposition of aerosol particles.

An *in vitro* model for aerosol deposition and transport across epithelia in the human airways may be a good predictor of therapeutic efficacy. An Andersen viable cascade impactor was used as a delivery apparatus for the deposition of particles onto monolayers of Calu-3 cells (Cooney et al., 2004). It was shown that these cell layers can withstand placement in the impactor, and that permeability can be tested subsequent to removal from the impactor. A simple, multi-stage cascade impinger is also a suitable physiologically-relevant model of the pulmonary epithelial barrier that would allow for quantitative characterisation of therapeutic aerosols *in vitro* (Fiegel et al., 2003). Calu-3 cells grown under air-interfaced culture (AIC) on a semipermeable membrane were impinged with aerosolised large porous particles. These particles were deposited homogeneously and reproducibly on the cell surface and caused no apparent damage to cell monolayers as evaluated by SEM and light microscopy. The monolayers showed no significant change in barrier properties within the first 90 min following particle application. This *in vitro* model, based on AIC grown Calu-3 cells, could allow a more relevant and quantitative characterisation of therapeutic aerosol particles intended for delivery to the tracheobronchial region of the lung or to the nasal passages.

Outlook

The "air-blood barrier" is not only relevant for the safe and efficient delivery of drugs, but is also subject to exposure to various air-borne pollutants in the form of (nano) particles. Therefore, the lung plays a major role as a target organ in any systemic toxicity test (acute, subchronic, and chronic) as well as in inhalation toxicology or respiratory sensitisation. In all these areas suitable alternatives to animal experiments are desperately sought.

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