Toxicology in Vitro 30 (2015) 217–224

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Biokinetics in repeated-dosing in vitro drug toxicity studies

Nynke I. Kramer [a,*], Emma Di Consiglio [b], Bas J. Blaauboer [a], Emanuela Testai [b]

[a] Institute for Risk Assessment Sciences, Utrecht University, Utrecht, The Netherlands
[b] Mechanism of Toxicity Unit, Environment and Primary Prevention Department, Istituto Superiore di Sanità, Rome, Italy

ARTICLE INFO

Article history:
Received 20 May 2015
Revised 11 August 2015
Accepted 4 September 2015
Available online 8 September 2015

Keywords:
In vitro
Repeated dosing
Quantitative in vitro–in vivo extrapolation
Biokinetics
Predict-IV

ABSTRACT

The aim of the EU FP7 Predict-IV project was to improve the predictivity of in vitro assays for unwanted effects of drugs after repeated dosing. The project assessed the added benefit of integrating long-lived in vitro organotypic cell systems with 'omics' technologies and in silico modelling, including systems biology and pharmacokinetic assessments. RPTEC/TERT1 kidney cells, primary rat and human hepatocytes, HepaRG liver cells and 2D and 3D primary brain cultures were dosed daily or every other day for 14 days to a selection of drugs varying in their mechanism of pharmacological action. Since concentration–effect relationships not only depend on the activity of the drug or the sensitivity of the target, but also on the distribution of compounds in the in vitro system, the concentration of a selection of drugs in cells, microtitre plate plastic and medium was measured over time. Results, reviewed in this paper, indicate that lipophilic drugs bind significantly to plastic labware. A few drugs, including less lipophilic drugs, bind to cell-attachment matrices. Chemicals that reach high concentrations in cells, including cyclosporin A and amiodarone, significantly accumulate over time after repeated dosing, partly explaining their increased toxicity after repeated dosing, compared to a single dose.

1. Introduction

Many derivatives of a lead compound exhibiting a desired pharmacological effect are synthesised early on in the development of a new pharmaceutical entity in order to identify the ones with the most optimal pharmacological response. In order to assess the safety of these lead compounds before the 'first dose in man', toxicity testing of a large number of chemicals on animals is necessary and a costly endeavour (Sasseville et al., 2004). This testing represents one of the major bottlenecks in drug development as toxicity testing in preclinical studies is time consuming and requires large numbers of animals and considerable amounts of test compound. In addition, the high costs of toxicity testing are exacerbated by the high drug attrition rate, where 23% of registered drugs are retracted due to adverse reactions not predicted in animal models (Kola, 2008). These adverse reactions are often idiosyncratic and occur after repeated dosing. Major reasons for the suboptimal correlation between animal and human toxicity are the inter- and intra-species differences in pharmacokinetics (Park et al., 2011).

In light of both ethical and financial costs associated with drug safety testing on animals, human cell-based in vitro assays are increasingly used to screen drug candidates for human-relevant pharmacokinetic properties and molecular mechanisms of toxicity prior to pre-clinical testing in animals. However, the move from using in vitro assays for hazard identification, i.e. the mere potential of a chemical to cause an effect, to hazard characterization in drug development, i.e. dose–response assessment, is still in its early stage of development. It is generally accepted that no single stand-alone in vitro test sufficiently replaces an animal-based toxicity test and thus an integrated strategy is required. Such strategy calls for a battery of in vitro assays employing long-lived, highly functional organotypic cell cultures and a mechanistic understanding of the molecular events leading to adverse health effects (Adler et al., 2011). For such in vitro test battery to be used in a risk assessment procedure, a point of departure needs to be derived from the set of dose–response relationships obtained from these assays and translated into a toxicologically equivalent dose in humans. Indeed, the pharmacokinetics (i.e. the absorption, distribution, metabolism and excretion from a body) of a drug determines the concentration over time of the drug (or its toxicologically relevant metabolite) at the target site, which strongly dictates the drug's toxicity. These processes need to be integrated into a meaningful in vitro-based drug safety testing strategy (Adler et al., 2011).

To improve the predictivity of in vitro systems for unwanted effects of drugs after repeated dosing, the aim of the EU 7th Framework Project, Predict-IV, was to develop such a testing strategy integrating in vitro systems with knowledge of cell biology, mechanistic toxicology and in silico (pharmacokinetic) modelling. The project focussed its efforts on developing testing strategies by using in vitro assays with cells of human origin (whenever possible) and representing target organs most frequently affected by

* Corresponding author at: Institute for Risk Assessment Sciences, Utrecht University, P.O. Box 80177, 3508 TD Utrecht, The Netherlands. Tel.: +31 30 253 5314.
E-mail address: N.I.Kramer@uu.nl (N.I. Kramer).

http://dx.doi.org/10.1016/j.tiv.2015.09.005
0887-2333/

poorly predicted drug toxicity, namely the liver, kidney and central nervous system. It opted for using primary human hepatocytes, primary rat hepatocytes and the human hepatoma cell line HepaRG as its main liver models (Mueller et al., 2015), 2D mouse and 3D rat primary brain cell models with an in vitro blood brain barrier (BBB) model to predict neurotoxicity (Culot et al., 2008, 2013; Schultz et al., 2015), and the human renal proximal tubule cell line RPTEC/TERT1 (Wieser et al., 2008; Aschauer et al., 2015b) as its model of choice for predicting nephrotoxicity. Culture conditions were adapted to maintain highly differentiated organotypic cells in culture for 14 days, during which the cells were exposed daily to a selection of 27 drugs varying in their mechanism of pharmacological action and known to cause hepatotoxicity, nephrotoxicity and/or neurotoxicity after repeated use. For a holistic, mechanistic approach, in depth characterization of molecular perturbations induced by the drugs was performed by integrating a suite of 'omics' technologies (e.g. Wilmes et al., 2013). Moreover, exposure conditions and changes within the assays over the 14-day exposure period were monitored and modelled (e.g. Pomponio et al., 2015a,b; Truisi et al., 2015). Dose response analyses and physiologically based pharmacokinetic (PBPK) models were developed to relate daily oral exposure to in vitro derived points of departures (Hamon et al., 2015).

2. Role of in vitro biokinetics in quantitative in vitro–in vivo extrapolation (QIVIVE) studies

Predict-IV uniquely devoted a separate work package (WP3 'Non animal-based models for in vitro kinetics and human kinetic prediction') to propose and apply a step-wise strategy to measure and model cell exposure levels over time of a selected number of drugs in the developed in vitro assays. The aim was to assess whether and how knowledge of the kinetics of drugs in in vitro assays helps to explain the variations in observed effects between drugs, cell types and assay setup, and – in so doing – improve the predictive value of in vitro observed effect concentrations. The inclusion of this work package was based on an increasingly acknowledged problem that the in vitro nominal concentration, the concentration in medium that is added to the cells in vitro and traditionally used to express in vitro concentration–effect relationships, is not necessarily proportional to the biologically effective dose (BED), the concentration at the target site inside the cells, across in vitro assays and chemicals, and between in vitro and in vivo systems (Groothuis et al., 2015). The BED is most closely related to the initial molecular changes caused by the drug in the cell and may represent only a fraction of the nominal concentration (Escher and Hermens, 2004; Paustenbach, 2000). A drug added to an in vitro test system may significantly bind to serum constituents such as albumin and lipids (Seibert et al., 2002), sorb to the plastic of a microtitre plate (Kramer et al., 2012), or evaporate into the headspace (Knöbel et al., 2012; Tanneberger et al., 2013). These processes reduce the in vitro bioavailability of the drug, which determines its concentration at the target site. Moreover, assays may vary in their assay setup (e.g. medium composition) and chemicals may vary in their affinity for in vitro system components, explaining differences in target concentrations across chemicals and assays despite similar concentrations added to the systems (Armitage et al., 2014; Kramer et al., 2012). As such, the nominal concentration in medium is not an adequate indicator of exposure to cells when interpreting and comparing in vitro toxicity data for different chemicals and between different in vitro systems. Indeed, concentration–effect relationships not only depend on the activity of the drug or the sensitivity of the target, but also on the distribution of compounds in the in vitro system (Gülden and Seibert, 2003).

Previous studies have generally focussed on assessing the distribution of chemicals in in vitro systems done after single exposures in simple, metabolically inactive cytotoxicity assays (Gülden and Seibert, 2003; Kramer et al., 2012; Stadnicka-Michalak et al., 2014; Tirelli et al., 2007). It is generally assumed that the test chemical in these assays reaches a chemical equilibrium between the exposure medium, well plate plastic and cell concentration, from which the freely available concentration, generally considered independent of assay setup and more closely related to the BED than the nominal concentration, can be ascertained (Armitage et al., 2014; Gülden and Seibert, 2005; Kramer et al., 2012). However, the assays developed in the Predict-IV project consisted of highly differentiated cells, differing in their metabolic competence and expression of transporters, and dosed repeatedly with test chemicals over a period of 14 days. Simple chemical equilibrium models described in literature estimating the distribution of the drugs are unlikely to suffice. Indeed, differences in observed (cytotoxic) effects between single and repeated dosing may be attributable to an accumulation of the chemical (or its metabolites) in the cells over time, after repeated dosing. In addition, differences in observed effects between cell types, e.g. in vitro kidney, liver and brain models, may be attributable to differences in the uptake and efflux of drugs into cells and its metabolic activation and clearance. By not understanding these differences in distribution of drugs in the Predict-IV in vitro test battery hampers the extrapolation of the observed effects to the in vivo system, where accumulation in cells over time may vary significantly from the in vitro situation. To assess the role of the in vitro distribution of drugs in explaining differences in the toxic potential of drugs across the assays tested in the Predict-IV project, the distribution of a selected number of drugs was measured in kidney, liver, brain and intestinal absorption models (Table 1). The results of these studies are reviewed in this paper.

3. Extracellular concentrations of drugs over time

3.1. Chemical stability

Chemical stability in solution determines the concentration in cells and subsequently its potential to perturb molecular pathways in vitro. The concentration in stock solutions and exposure medium of drugs listed in Table 1 were measured over time. Whereas most drugs were chemically stable in exposure medium as well as in the vehicles used to prepare stock solutions, i.e. distilled water, methanol and DMSO, adefovir dipivoxil hydrolysed significantly in exposure medium of RTPEC/TERT1 cells at 37 °C, hampering the interpretability of their in vitro effect concentrations. Less than 15% of the parent compound was left in solution, highlighting the importance of assessing the effect of hydrolysis products in addition of parent drugs in in vitro toxicity assays (Crean et al., 2015). Amiodarone significantly hydrolysed in distilled water, but not in exposure medium, methanol or DMSO. Stock solutions for amiodarone were therefore only prepared in methanol or DMSO, which were used to directly spike the exposure medium (Pomponio et al., 2015a,b). Data from the Predict-IV project highlight the benefit of measuring the drug concentrations in both stock and working solutions to avoid aberration in cell treatment. The nominal concentration differed from the measured concentration in exposure medium up to 30% for amiodarone and ibuprofen (Pomponio et al., 2015a; Truisi et al., 2015) and even greater differences were reported for cyclosporine A in treating neuronal cells (Bellwon et al., 2015b). As opposed to be being solely attributable to biological variation, variations in effect concentrations between replicate experiments may be attributable to inconsistencies between nominal and measured concentrations of drugs in exposure medium added to in vitro assays.

3.2. Sorption to plastic

Sorption to in vitro system components such as plastic labware and microtitre plates was shown to significantly reduce the freely available drug concentration in in vitro assays for a number of lipophilic drugs. Up to 60% of amiodarone, one of the most lipophilic drugs tested in WP3 of the Predict-IV project, with a $\log D_{7.4}$ of 3.4, was found to bind to plastic labware (Pomponio et al., 2015a). The drug was also found to significantly bind to microtitre plate plastic in a dose- and time-dependent manner (15–35%), suggesting plastic binding saturates at higher concentrations of the drug. The extent of plastic binding was reduced in the presence

Table 1
Drugs and their physicochemical properties tested for their distribution in in vitro systems within the Predict-IV project.

Drug	Therapeutic class	% bound in human plasma[a]	Experimental LogP[a]	Log $D_{7.4}$[b]	Assays used for biokinetics analyses (reference)
Chlorpromazine	Antipsychotic, neuroleptic	97.8%	5.41	3.39 (basic)	Single and repeated dose exposure brain, liver, intestinal models (Broeders et al., 2012, 2013, 2015a,b)
Amiodarone	Antiarrhythmic	99.98%	7.80	5.66 (basic)	Single and repeated dose exposure liver, brain models (Pomponio et al., 2015a,b)
Diazepam	Anxiolytic, anticonvulsant, tranquilizer	99%	2.82	Neutral	Repeated dose exposure brain model (Broeders et al., 2015a)
Cyclosporin A	Immunosuppressor	90%	2.95	Neutral	Repeated dose exposure kidney, liver, brain model (Wilmes et al., 2013; Bellwon et al., 2015a,b)
Cisplatin	Anticancer, antineoplastic	97.5%	−2.35	Neutral	Repeated dose exposure kidney model (Wilmes et al., 2015)
Colchicine	Anti-inflammatory	23%	1.30	Neutral	Single and repeated dose exposure BBB model (Fabulas-da Costa et al., 2013)
Adefovir dipivoxil	Anti-viral	4% [c]	2.45[c]	Neutral	Repeated dose kidney model (Crean et al., 2015)
Ibuprofen	Anti-inflammatory	99% [d]	3.97[e]	0.8 (acidic)[e]	Single and repeated dose exposure liver model (Truisi et al., 2015)

[a] Unless otherwise stated, drug properties are taken from Fabulas-da Costa et al. (2013).
[b] Seydel and Wiese (2002).
[c] Dörwald (2012).
[d] Paliwal et al. (1993).
[e] Avdeef et al. (1998).

of more than 2% foetal bovine serum (FBS) in the exposure medium because serum constituents and plastic compete for binding the drug (Pomponio et al., 2015a). Amiodarone's less lipophilic metabolite, mono-N-desethyl-amiodarone (MDEA), was found to adsorb significantly less (5–10%) to plastic (Pomponio et al., 2015a,b). Indeed, sorption of non-lipophilic chemicals, including ibuprofen with a log $D_{7.4}$ of 0.8, adefovir dipivoxil with a log P of 2.45 and cisplatin with a log P of −2.35, did not significantly reduce the freely available concentration (Crean et al., 2015; Truisi et al., 2015; Wilmes et al., 2015). These findings are in line with studies correlating the lipophilicity of neutral organic chemicals with the extent to which the chemical binds to plastic microtitre plates and the difference between the nominal and freely available concentration in serum-free exposure medium (Armitage et al., 2014; Riedl and Altenburger, 2007; Stadnicka-Michalak et al., 2014).

In 14-day repeated dose cytotoxicity assays with RTPEC/TERT1 cells in 6-well plates, cyclosporine A, with a log P of 2.95, was found to bind rapidly to well plate plastic and microporous inserts. A maximum of 12% was bound to plastic within 24 h after dosing the medium with 5 μM and 15 μM of the drug (Wilmes et al., 2013). This percentage did not change over the 14-day exposure period, indicating that no accumulation to plastic occurred. Similar behaviour was reported for cyclosporine A in 2D rat brain cultures in 6-well plates (Bellwon et al., 2015a). The extent of plastic binding, however was lower peaking at 6.5% of the total amount in the well at 0.1 μM concentrations and 3.4% at 2 μM concentrations in serum-free exposure medium. The lower extent of binding to plastic in these neuronal cell assays compared to the kidney cell assays is likely due to the lack of microporous inserts in the former, reducing the surface area of plastic to which the chemical can bind. Indeed, in the absence of cells, effectively exposing greater plastic surfaces, cyclosporine A bound over 70% to the microporous inserts and well plate plastic (Bellwon et al., 2015a). The decrease in the extent of binding at higher medium concentrations of cyclosporine A in 2D rat brain cultures also suggests that plastic binding of the chemical is saturable at higher cyclosporine A concentrations in these assays.

Like amiodarone and cyclosporine A, lipophilic chlorpromazine, with a log $D_{7.4}$ of 3.39, also significantly adsorbed to microtitre plate plastic. In a Caco-2 cell transport assay, up to 25% of chlorpromazine was bound to the plastic of the microporous inserts and well plate (Broeders et al., 2012). The binding to plastic was found to be rapid and the extent of binding remained constant over the 60-minute assay. Similar to amiodarone, plastic binding of chlorpromazine in a Caco-2 transport assay was saturable, as the percentage of chlorpromazine bound to plastic decreased with increasing concentrations in medium. Interestingly, the extent of plastic binding also depended on the site of application of chlorpromazine. When dosed apically, the percentage of chlorpromazine bound to plastic was twice that bound to plastic when dosed basolaterally, which may be explained by the greater surface area (of plastic) to volume (of medium) ratio in the apical compartment compared to the basolateral compartment (Kramer et al., 2012). The extent to which chlorpromazine adsorbed to well plate plastic also depended on the presence of bovine serum albumin (BSA), the main drug-binding protein in FBS. As previously described in Kramer et al. (2012) and Schirmer et al. (1997) for phenanthrene and fluoranthene in basal cytotoxicity assays, the percentage of chlorpromazine in a Caco-2 transport assay bound to plastic was insignificant (<10%) in the presence of physiological (600 μM) levels of BSA as the chemical preferentially associated with BSA instead (Broeders et al., 2012). On the other hand, lower concentrations of BSA in exposure medium (e.g. 10 μM BSA corresponding to approximately 2% FBS) did not affect the extent of plastic binding in a cytotoxicity assay of chlorpromazine with HepaRG cells in 6-well plates (Broeders et al., 2015b). Moreover, in cytotoxicity assays with primary rat and human hepatocytes in 6 well plates, in the presence or absence of low concentrations of BSA, no plastic binding of chlorpromazine was observed (Broeders et al., 2015b). It is unclear why plastic binding was up to 25% in HepaRG cytotoxicity assays and Caco-2 transport assays and undetectable in primary hepatocyte cytotoxicity assays, but the presence of cell-attachment matrices (collagen and Geltrex™) in the primary hepatocyte cultures may have blocked much of the surface area of well plate plastic for sorbing chlorpromazine as well as served as an alternative sorption site.

3.3. Binding to cell-attachment matrices

The relationship between a drug's lipophilicity and binding to cell-attachment matrices is not as clear cut as it is for binding to plastic labware. To maintain hepatocyte functions in culture for the 14-day repeated dose exposure experiments performed within the Predict-IV project, primary rat hepatocytes were seeded onto a collagen I layer in 6-well plates (Bellwon et al., 2015b; Broeders et al., 2015b; Truisi et al, 2015). Primary human hepatocytes were also seeded onto a collagen I layer in 6-well plates, but additionally covered with Geltrex™, which was renewed every 3–4 days (Bellwon et al., 2015b; Broeders et al., 2015b; Pomponio et al., 2015a; Truisi et al, 2015). For ibuprofen, chlorpromazine, cyclosporine A and amiodarone, the extent of binding to cell-free collagen and/or Geltrex™ was analytically measured and factored in when determining cell-associated concentrations of the drugs. Ibuprofen, a drug with a low lipophilicity and insignificant binding to well plate plastic, significantly bound to collagen in a time-dependent manner (Truisi et al., 2015). The time-dependence of this binding process suggests that ibuprofen accumulates in the collagen after repeated dosing. Ibuprofen levels in collagen

increased from 15% of the total amount of ibuprofen added after the first day of dosing (day 0) to 30% on day 13. The concentration in exposure medium (10 μM and 100 μM ibuprofen) had no effect on the percentage of ibuprofen bound to collagen. Interestingly, ibuprofen did not bind to Geltrex™. The more lipophilic chlorpromazine and cyclosporine A also did not bind to Geltrex™, whereas both bound to a similar extent and time-dependent manner to collagen (Bellwon et al., 2015b; Broeders et al., 2015b). Only amiodarone was found to bind to Geltrex™, but this binding was only measurable after 13 days of daily-repeated dosing and accounted for only 5–10% of the total dose applied (Pomponio et al., 2015a). All in all, Predict-IV data suggest that the quantitation of the possible physical sequestration of drugs, especially during repeated dosing, is crucial to avoid overestimating cell-associated concentrations. Concentrations of drugs associated with cells and concentrations associated with cell-attachment material are not easily discerned in the same assay, as it is difficult to separate the cells from cell-attachment material.

3.4. Binding to serum constituents

Binding to serum constituents in exposure medium, particularly binding to serum albumin, has been shown previously to be one of the most significant contributors to the reduction in the freely available concentration and hence cell uptake of test chemicals in in vitro assays, explaining differences between observed effective concentrations between cell assays with different in vitro setups (Armitage et al., 2014; Groothuis et al., 2015; Gülden and Seibert, 2005). For neutral organic chemicals, the binding affinity to albumin is positively correlated with the lipophilicity of the chemical (Armitage et al., 2014; DeBruyn and Gobas, 2007; Endo and Goss, 2011). Other properties, like charge and size, also dictate the extent of plasma binding (Austin et al., 2005; Gülden and Seibert, 2005; Kratochwil et al., 2004). Anions have a stronger affinity to albumin than cations, which are known to bind preferentially to serum glycoproteins (Kratochwil et al., 2004). In the Predict-IV project, a significant number of lipophilic neutral, acidic and basic drugs, including chlorpromazine, amiodarone, diazepam, cyclosporin A and ibuprofen, were used as test chemicals and are known to strongly bind to human plasma constituents (Table 1). The type and level of serum used in the assays selected for the Predict-IV project originally varied between assays and from human plasma. Since this variation could hamper effect concentration comparisons and toxicity ranking, Predict-IV developed and used, where feasible, serum-free assays for comparing effect concentrations. The only serum-containing assay used was the 14-day hepatotoxicity assay with HepaRG, where 2% FBS was deemed the minimum level of serum necessary to maintain the cell line's hepatocyte morphology and function. Yet assuming a one-to-one binding ratio with albumin, albumin binding was likely to be near saturation at the test chemical concentrations tested in these assays and thus the effects on serum constituent binding on the effect concentrations measured in these assays were considered negligible. This was confirmed by results obtained with amiodarone by using different FBS concentrations in HepaRG cells. With 0 and 2% FBS in medium, the extent of cell uptake of 1–5 μM amiodarone was very similar, whereas at 10% FBS cell uptake of amiodarone was lower and slower, being reduced by 50% following a single treatment (Pomponio et al., 2015a). This clearly underlines that the same nominal concentrations cannot be expected to give the same toxicological outcome when different FBS concentrations in the medium are used as FBS alters the bioavailability of the drug. The nominal concentration is therefore a poor dose metric for highly serum constituent-bound drugs.

Given that the use of serum-free assays is not always feasible, developing robust methods to estimate drug binding to plasma proteins in in vitro toxicity assays is important. Several methods are commonly used to estimate plasma protein binding affinities, including equilibrium dialysis, ultrafiltration and ultracentrifugation. However, these techniques are often unsuitable for testing the binding affinity of lipophilic drugs, like those used within the Predict-IV project, because the drugs may strongly bind to membranes and desorb from protein, resulting in a change in the equilibrium between bound and unbound drugs (Heringa and Hermens, 2003). One aim within the Predict-IV project was therefore to assess the feasibility of solid phase microextraction (SPME) to measure serum constituent binding of drugs in in vitro exposure medium. Broeders et al. (2011) describes a negligible depletion (nd)-SPME method to measure the freely available concentration of chlorpromazine in in vitro exposure medium containing BSA. The optimised method consists of 2 cm glass fibres coated with 7 μm thick polyacrylate, which quickly (within 3 h) sorb less than 5% of the unbound chlorpromazine in in vitro exposure medium supplemented with BSA or serum, thus not disturbing the chemical equilibrium between bound and unbound chlorpromazine. The chemical is subsequently extracted from the fibre to analytically determine its concentration, which is linearly related to the freely dissolved concentration in the exposure medium up to 1 mM by the chemical's fibre–water partition coefficient, 717 L/L. Using the nd-SPME, an affinity constant for chlorpromazine to BSA of 18,355 L/mol at physiological serum albumin levels was calculated, indicating that 92% of chlorpromazine in serum is protein-bound. This is in line with in vivo literature findings (Broeders et al., 2011). It also illustrates that at serum levels typically found in vitro (5–20% v/v), less than 50% of the chlorpromazine is protein-bound. By accounting for the difference in the unbound fraction of the chemical between in vitro exposure medium and in vivo plasma, the nd-SPME method allows for a more direct comparison of in vitro effect concentration to in vivo plasma concentrations used in PBPK models for QIVIVE purposes (Louisse et al., 2010).

Within the Predict-IV project, the nd-SPME method developed by Broeders et al. (2011) was used to investigate the influence of albumin binding of chlorpromazine in various in vitro assays, including the intestinal permeability assay with the commonly used human colorectal carcinoma cell line, Caco-2 (Broeders et al., 2012). Albumin is occasionally added to the basolateral compartment of the assay to mimic proteins in blood (Hubatsch et al., 2007). However, the calculation of permeability coefficients requires the estimation of unbound concentrations of the test compound, as well as negligible accumulation of the compound in cells and plastics, conditions which are often overlooked (Hubatsch et al., 2007). In the case of chlorpromazine, 94% was bound to albumin when 600 μM BSA was added to the basolateral compartment. Depending on the level of albumin in the system and the direction of transport measured, $\leq$20% was found in cells and $\leq$25% was bound to plastic (Broeders et al., 2012). The low recovery of free chlorpromazine in the medium compartments over the exposure period indicated that the calculation of an apparent permeability value (Papp) is infeasible. The calculated Papp varied significantly with in vitro setup (e.g. transport direction, BSA concentration, time of measurement and chlorpromazine concentration).

Following up on the aforementioned Caco-2 study, Broeders and co-workers further investigated the influence of serum protein binding on the apparent sensitivity of Balb/c 3T3, Caco-2 and HepaRG cells to chlorpromazine cytotoxicity (Broeders et al., 2013). Cytotoxic concentrations were expressed using different dose metrics, including the nominal, measured total (accounting for cell and plastic binding) and measured free (accounting for cell, plastic and serum protein binding by using nd-SPME) chlorpromazine concentrations in medium. Results indicated that the ranking of sensitivity of the three cell types to chlorpromazine toxicity was dependent on the dose metric. When ranking the cell types according to the median effect concentrations (EC_{50}) based on nominal concentrations, Balb/c 3T3 cells were most sensitive to chlorpromazine toxicity (EC_{50} 8 μM), followed by HepaRG cells (EC_{50} 20 μM) and Caco-2 cells (EC_{50} 79 μM). However, when ranking the cell types by EC_{50} based on freely dissolved medium concentrations, Caco-2 cells were more sensitive to chlorpromazine toxicity than HepaRG cells (21 versus 28 μM) and its EC_{50} was only 2 instead of 9 times higher than that of Balb/c 3T3. One reason for this discrepancy in ranking is that typical Caco-2 cytotoxicity assays contain 10% serum and thus only 20% of the test chemical is not bound to serum constituents and is available for uptake into cells, whereas 70% is unbound in a Balb/c 3T3 assay with 5% serum

in assay medium and 100% is unbound in HepaRG assay medium, as it does not contain serum. Up to 90% of the chemical added to the assay was found in HepaRG cells, whereas 50% was found in Caco-2 cells and 10% was found in Balb/c 3T3 cells at non-cytotoxic concentrations. A higher free concentration in medium of the Balb/c 3T3 cytotoxicity assay compared to the Caco-2 assay would suggest that, if cell numbers are equal, a greater fraction of the drug would partition into Balb/c 3T3 cells compared to Caco-2 cells. Broeders et al. (2013), however, found the opposite. The authors attribute this to the fact that the Caco-2 assay contained 6 times the amount of cell protein, a proxy for cell number, than the Balb/c 3T3 assay at the start of the exposure. The studies within the Predict-IV projects comparing effects of different drugs in different cell systems effectively illustrate that differences in sensitivity between cell systems based on nominal or even measured drug concentrations do not represent the differences in intrinsic sensitivity of the assay. A large part of the aim of the Predict-IV project was to compare the intrinsic sensitivity of organotypic cell assays to drugs to discern target organs of drugs. Therefore, one outcome from the Predict-IV project is that the use of free medium concentration or the cell-associated concentrations of drugs over time represents a better dose metric for comparing in vitro assays than the traditionally used nominal concentration.

4. Intracellular concentrations of drugs over time

4.1. Expression and activity of drug transporters and biotransformation enzymes

To identify specific mechanisms of toxicity and new toxicity pathways, it is important to distinguish between an increase in cytotoxic potency of a drug in vitro that is due to higher intracellular concentrations of the drug (i.e. due to differences in kinetics) and an intrinsic sensitivity of a cell type to a drug (i.e. the drug's ability to activate selective toxicity pathways). If a significant fraction of a drug is taken up by cells, repeated dosing of the cells, as done in the Predict-IV project, could lead to an increase in the total amount of drug in the system over time and an accumulation of the chemical in cells specifically. On the other hand, if clearance of the drug is significant between the dosing steps, accumulation of the drug (or its metabolites) in cells may be minimal, but the accumulation of the damage can occur. Differences in accumulation over time (or more generally in kinetics) may explain possible difference in cytotoxic potency of drugs between single and repeated dosing, differences in cytotoxic potency between drugs after repeated dosing and differences in sensitivity between cell types.

For many organic chemicals, the concentration in cells is proportional to the free concentration and is dictated by its lipophilicity, e.g. logP or $logD_{7.4}$ (Armitage et al., 2014; Austin et al., 2005; Gülden and Seibert, 2003). One could use and build on existing quantitative structure–activity relationships (QSARs) and related equilibrium partition models linking (free) medium concentrations with cell and tissue concentrations and toxicity (Armitage et al., 2014; Gülden and Seibert, 2003; Stadnicka-Michalak et al., 2014). However, a number of drugs, including those tested in the Predict-IV project, are actively taken-up by or excluded from cells via selective transporters, or metabolically activated or degraded by cells (e.g. Broeders et al., 2012; Wilmes et al., 2013). In these cases, concentrations in cells may not follow predictions of cell concentration in time based on partitioning. Whether such transport and biotransformation occur is dependent on the drug as well as the cell systems used. Many cell lines lack transporters and biotransformation enzymes, and when they do, they are poor surrogates of in vivo toxicity regulated by these transporters and enzymes (Webborn et al., 2007).

Within the Predict-IV project, the level of expression and activity of drug transporters and biotransformation enzymes were assessed over the 14-day exposure period for each liver, kidney and brain model. Aschauer et al. (2013, 2015a) have shown that RPTEC/TERT1 cells exhibit phenotypic biomarkers of differentiated proximal tubule cells. Upon contact inhibition, the cells are characterized by a decreased glycolysis rate, an increased capacity for oxidative respiration, and an increased expression of proximal tubule tight junction proteins, cilia associated proteins and drug transporters, including organic cation transporters (OCT-2, -3 and -N2), MATE-1 and -2, organic anion transporters (OAT-1, -3 and -4), and ABC transporters (p-glycoprotein, MRP2, MRP4, BCRP and ABC-C5). The functionality of the OCTs was demonstrated by directional transport with fluorescent dye 4-Di-1-ASP. No major changes in transcriptome occurred over the exposure time. Differentiated RPTEC/TERT1 cells were also found to be less sensitive to cyclosporine A toxicity than undifferentiated RPTEC/TERT1 cells, suggesting that P-glycoprotein (PgP) actively extruded the drug in differentiated cells.

Vichi et al. (2015) report the constitutive expression of cytochrome P450 CYP1A1, CYP2B1/B2, CYP2D4, CYP2E1 and CYP3A isoforms at the mRNA and protein levels in astrocytes, neurons and to a minor extent in oligodendrocytes in the 3D aggregating rat brain cultures. The expression levels and cell-specific localization of these cytochrome P450 enzymes, known to metabolize a number of centrally acting xenobiotics, appear comparable to in vivo rat brains (Ravindranath and Strobel, 2013). The activity of these enzymes was further demonstrated by Pomponio et al. (2015a). The authors report the detection of mono-N-desethylamiodarone (MDEA), the major oxidative metabolite of amiodarone after 14 days of treatment.

The expression, functionality and inducibility of biotransformation enzymes and transporters in primary rat and human hepatocytes cultured in a sandwich configuration for 14 days have been well described in literature (Hewitt et al., 2007) and summarised elsewhere in this special issue (Mueller et al., 2015). Generally, rat hepatocytes have a fairly constant activity of drug metabolizing enzymes. Human hepatocytes from different donors exhibit a large variation in metabolizing enzyme levels. To help interpret variations in toxicity to human hepatocyte cultures, CYP levels were determined in control and drug-treated hepatocyte cultures 4 times over the 14-day exposure period. The long-term viability and organotypic functionality of HepaRG cells over a 14-day experiment have been demonstrated prior to the Predict-IV project (Anthérieu et al., 2010; Jossé et al., 2008; Lübberstedt et al., 2011). The cells express most CYPs, phase II enzymes and transporters at levels comparable to cultured primary human hepatocytes and in vivo, making them ideally suited for long-term in vitro clearance and hepatotoxicity studies. Within Predict-IV, Savary et al. (2015) evaluated the long-term functional stability of the cell line and their response to drugs after repeated dosing using a transcriptomic approach. The authors found less than 1% of the expressed genes to be markedly altered over the 14-day period. These alterations included a down-regulation of mainly cell cycle-related genes and an overexpression of genes involved in xenobiotic and lipid metabolism, which was supported by the results showing a treatment-unrelated increase in CYP activity over 14 days (Pomponio et al., 2015a; Truisi et al., 2015).

4.2. Measuring and modelling intracellular concentrations over time

Because metabolism and transporter activity may influence the accumulation of both lipophilic and non-lipophilic drugs differentially across the liver, kidney and brain models, the concentration in cells of drugs with increasing lipophilicity (cisplatin, adefovir dipivoxil, diazepam, cyclosporine A, ibuprofen, chlorpromazine and amiodarone) was measured in one or more cell models on day 0 (first day of treatment) and day 13 at five different time points after dosing, selected on the basis of the drug and cell system characteristics. Cells were dosed repeatedly (i.e. daily for liver and kidney models, every other day for brain models) to two concentrations of the drug (i.e. approximately 10% viability concentration, EC_{10}, and 1/10 EC_{10} after 14 days of treatment), in order to follow the kinetic behaviour of the test compound over time in the absence of significantly changing cell numbers. Indeed, changing cell numbers in vitro is known to affect observed effect concentrations (Gülden et al., 2001, 2015). For a selection of drugs, cells were simultaneously harvested at days 1, 3 and 14 to inspect transcriptomic, proteomic and metabolomic profiles. Since one of the goals of the Predict-IV project was to also predict potential cellular accu-

mulation of drugs from the concentration–time profiles after the first dose (day 0) and link these predictions to omics variations, several variants of a three-compartment model were established to describe the time course of the amounts of drugs (and metabolites) in medium, on plastic and in cells. The three-compartment model and model output are reviewed elsewhere in this Special Issue (Hamon et al., 2015). The sampling strategy used in the Predict-IV project was an attempt to optimise the balance between the number of samples generated and the amount of information obtained. However, once the drug concentrations in the various in vitro compartments were measured and used as model input, it became clear that for some drugs (e.g. those showing the potential to accumulate in cells) the availability of at least one more intermediate sampling time point between day 0 and day 13 would have been beneficial and reduced model parameter variation. Similarly, assessing the kinetics of more than two drug concentrations may have provided greater mechanistic insights into saturation processes.

A non-lipophilic drug such as ibuprofen was rapidly up-taken by hepatic cells (Truisi et al., 2015). A dynamic equilibrium reached within 1 to 2 days. The kinetic profiles for ibuprofen dosed at 10 and 100 μM, the latter roughly corresponding to human therapeutic plasma concentrations, were similar. At any time point, the fraction of ibuprofen measured in cells was negligible compared to the fraction in the medium. However, higher intracellular concentrations were detected in primary rat hepatocytes than in human derived cells at steady state, which is likely due to the significantly lower metabolic clearance rates of ibuprofen in the rat model compared to the human-models. Despite the differences in clearance rates between the hepatic models tested, the drug was efficiently metabolized and very rapidly cleared in all models, so no intracellular accumulation of the parent drug was found on day 13. Twenty-four hours after dosing, no or almost no ibuprofen was recovered from the medium, plastic and cells. Indeed, mass balance measurements provided a useful insight into the fate of the drug. Monitoring the concentration–time profiles in exposure medium only (which were similar in the three hepatic systems) would not have picked-up the difference in uptake and metabolic clearance rates between the three models as the decrease in medium was not quantitatively related to the increase of ibuprofen in the cell fraction. Thus, although technically demanding, the measurement of the intracellular concentrations and the calculation of the relative distributions using mass balance values were crucial parameters to be monitored (Truisi et al., 2015).

Non-lipophilic adefovir dipivoxil showed a similar kinetic profile as ibuprofen in RPTEC/TERT1 cells (Crean et al., 2015). Under normoxic conditions, the fraction of the drug in cells was very low compared to the fraction in medium, no intracellular accumulation took place and there were no kinetic differences between the low and high dosing concentrations. However, under hypoxic conditions, an increase in intracellular concentrations and a corresponding increase in toxicity were observed. Given the measured and modelled kinetic data, the authors postulated a mechanism involving intracellular accumulation of adefovir dipivoxil as a result of hypoxia dependent down-regulation of efflux transporter proteins.

Like in the study of adefovir dipivoxil by Crean et al. (2015), measuring and modelling the distribution of the organometallic drug cisplatin in RPTEC/TERT1 cells also helped to postulate a new mechanism of toxicity (Wilmes et al., 2015). There was no change in the kinetics of the drug in the exposure medium over time. However, the drug significantly accumulated into the cells over time, with no apparent maximum reached over the 14-day exposure period. The influx into the cells was ten times slower basolaterally than apically. The authors postulated that the drug was taken up basolaterally by the transporter OCT2, transported to the apical membrane and released via MATE1 facilitated transport and active MRP2 transport.

Lipophilic drugs like amiodarone and chlorpromazine significantly accumulated in cells over time at both dosing concentrations in all tested assay types (Table 1). The uptake into cells occurred quickly, reaching a maximum at 1–3 h after dosing. A dynamic equilibrium between the concentration in cell and medium after repeated dosing was generally reached around 7 days after the initial dose. Pomponio et al. (2015a,b) measured amiodarone concentrations and its major metabolite MDEA in primary human hepatocytes, HepaRG, 2D mouse and 3D aggregating rat brain cell cultures. Uptake of amiodarone into hepatocytes was quicker than in brain cultures, which can be explained by the metabolism of the drug by the hepatocytes simultaneously occurring during the uptake phase of the drug into cells. More than half of the total drug added partitioned to the hepatocytes and brain cells after the first dose. HepaRG cells had a 100-fold higher metabolic clearance than primary hepatocytes, which also increased over the exposure period. Consequently, the levels of MDEA at the end of the repeated treatment reached higher values in the HepaRG cell line. The concentration in both hepatocyte types at day 0 was similar. However, at day 13, primary human hepatocytes contained higher levels of amiodarone than MDEA and the opposite was true for HepaRG cells. The higher levels of MDEA accumulated inside the HepaRG cells can explain the induction of phospholipidosis at the highest tested concentration, not observed in human hepatocytes (Pomponio et al., 2015a). Accumulation of amiodarone also occurred in brain cultures, especially 3D aggregated brain cultures (approximately 3-fold increase). The most striking difference between brain and hepatocyte cultures was the formation and accumulation of MDEA in hepatocytes (with approximately a 1000-fold higher cell concentration than medium concentration). Brain cultures only showed a minor presence of the metabolite on the last day of exposure at the highest dose tested.

Like amiodarone, more than half of the total chlorpromazine added partitioned to hepatocytes and brain cells after the first dose (Broeders et al., 2015a,b). The concentration of chlorpromazine in cells was 3 to 4 times higher on the last day of exposure than on the first day. Again similar to amiodarone, uptake of chlorpromazine in brain cells after dosing stabilised quickly, but did not decrease after a maximum was reached like in hepatocytes. Unlike amiodarone, however, primary hepatocytes cleared chlorpromazine more quickly than HepaRG cells.

Unlike amiodarone and chlorpromazine, cyclosporine A displayed a totally different kinetic behaviour in the different cell systems: it accumulated in the renal cells at very high levels only at the highest tested concentrations (15 μM) (Wilmes et al, 2013), indicating that those cells have a low metabolic capacity and that the transporter PgP is not able to extrude cyclosporine A at that concentration (possibly due to possible saturation or inhibition of the transporter). On the contrary, the drug was efficiently cleared by the human hepatocytes and HepaRG cells and did not accumulate into the cells over time (Bellwon et al., 2015b). In the brain cell systems, very low intracellular cyclosporine A uptake was measured (in accordance with in vivo data) and remained near-constant over the 14 day exposure period. However, the level of drug in the cells at the end of the treatment was enough to induce Cyp-B secretion, as a marker of cyclosporine A toxicity (Bellwon et al., 2015b). The analysis of the transport of cyclosporine A through different barriers including the blood–brain barrier, indicates that the major route of cyclosporine A uptake in the brain is via unknown active transporters and is limited by the active efflux pump PgP.

5. Conclusions: a strategy for future in vitro testing

The aim of the EU FP7 Predict-IV project was to improve the predictivity of in vitro assays for unwanted effects of drugs after repeated dosing. The project assessed the added benefit of integrating long-lived in vitro cell systems, representing the liver, the kidney and the central nervous system, with ‘omics’ technologies and in silico modelling, including systems biology and pharmacokinetic assessments. WP3 studied the distribution over time (i.e. kinetics) of a selection of drugs, varying in physicochemical properties and mechanism of action, within each in vitro model tested. By integrating measured effect data with measured kinetics data, WP3 provided an approach to assess drug-induced stress over time and distinguish between kinetics and dynamic effects of the drug. The work package established a step-wise strategy, whose critical steps were:

- The assessment of the chemical stability of the drug over time in working solutions and in the various in vitro setups.
  Nominal concentrations may significantly differ from measured concentrations over time of drug instable in solution. In case of significant hydrolysis of the test chemical (i.e. > 10% of the nominal concentrations), measures to reduce it should be considered on the basis of the causative factors (i.e. change of the solvents used for the stock solution) or a correct estimation of the loss should be taken into account.
- The determination of the extent of drug binding to labware.
  The sorption of a drug to labware reduces the concentration of the drug available to uptake into cells. Depending on the lipophilicity of the drug, drugs were found to sorb to the plastic of flasks and well plates, as well as membrane filters on which cells are seeded. Moreover, physical sequestration of drugs in cell-attachment matrices was also measured for a few drugs (e.g. cyclosporine A in collagen I). Measuring the extent of such sequestration is proposed to avoid overestimating the cell-associated concentration of drugs. In case more than 10% of a drug sorbs to labware, plastic devices should be replaced with non-sorbing labware (e.g. glassware). If replacement is not possible, it is necessary to quantify the sorption and factor the amount sorbed into effect concentrations.
- The estimation of the binding potential of drugs to macromolecules in exposure medium.
  Medium constituents such as serum may significantly reduce the bioavailable concentration in in vitro assays of highly plasma bound drugs. Knowledge of the composition of exposure medium is required. Whenever possible, serum-free media should be used or serum in medium should be reduced to a minimum (as was the case for the HepaRG cultures used in the Predict-IV project).
- The use of cell-associated drug concentration over time to link in vitro exposure to effect.
  The relevance of measuring the cell-associated concentration of a drug besides the free concentration in medium was demonstrated in a number of studies within the Predict-IV project (e.g. Truisi et al., 2015). The drug can accumulate within cells over time, which may partly explain enhanced toxicity observed after repeated dosing. The number of cells over time should remain constant or should be known, since cell number strongly affects observed effect concentrations.
- The assessment of metabolic and transporter activity in in vitro models.
  The knowledge of the metabolic competence of cells used is crucial for mechanistically interpreting a decrease over time in the parent drug concentration recovered from the various in vitro assay compartments (cells, medium, plastic/collagen adsorption). The kinetics of the major metabolite of amiodarone over time has been measured within the Predict-IV project, but results indicate the usefulness of having kinetic data on the other metabolites of amiodarone as well. The metabolic characterization of the model has been made available for the liver and kidney models as well as for the 3D CNS model. Results also point to the need to characterize the expression and activity of biotransformation enzymes and transporters from various single human donors. Such characterization allows for the assessment of variability among samples in metabolism, as well as the active uptake into and efflux from cells of drugs.
  All in all, studies performed in WP3 of the Predict-IV project clearly suggest that knowledge of the kinetics of a drug in vitro provides an explanatory value of the information obtained from in vitro toxicity assays. Integrating knowledge of the differences in concentration of drugs in cells in vitro over time between dosing regimens with knowledge of transporter and biotransformation enzyme function allows for the development of a mechanistic understanding of the observed in vitro toxicity, as has been illustrated for e.g. cyclosporine A (Wilmes et al., 2013). The next step is to assess the prediction of specific organ toxicity after repeated dosing of individual drugs by comparing human doses extrapolated from in vitro effect concentrations based on cell concentrations over time in the different Predict-IV cell models using reverse dosimetry PBPK modelling, developed in work package 5 of Predict-IV (Hamon et al., 2015).

Acknowledgements

The authors declare no conflicts of interest. This work was supported by the EU FP 7 Predict-IV programme (grant 202222). The authors are grateful to the many PhD students and scientists performing the work cited in this overview paper. The authors would also like to acknowledge the Doerenkamp Zbinden Foundation for financially supporting N.I. Kramer and B.J. Blaauboer at Utrecht University.

References

Adler, S., Basketter, D., Creton, S., Pelkonen, O., Van Benthem, J., Zuang, V., Andersen, K.E., Angers-Loustau, A., Aptula, A., Bal-Price, A., Benfenati, E., Bernauer, U., Bessems, J., Bois, F.Y., Boobis, A., Brandon, E., Bremer, S., Broschard, T., Casati, S., Coecke, S., Corvi, R., Cronin, M., Daston, G., Dekant, W., Felter, S., Grignard, E., Gundert-Remy, U., Heinonen, T., Kimber, I., Kleinjans, J., Komulainen, H., Kreiling, R., Kreysa, J., Leite, S.B., Loizou, G., Maxwell, G., Mazzatorta, P., Munn, S., Pfuhler, S., Phrakonkham, P., Piersma, A., Poth, A., Prieto, P., Repetto, G., Rogiers, V., Schoeters, G., Schwarz, M., Serafimova, R., Tähti, H., Testai, E., Van Delft, J., Van Loveren, H., Vinken, M., Worth, A., Zaldivar, J., 2011. Alternative (non-animal) methods for cosmetics testing: current status and future prospects — 2010. Arch. Toxicol. 85 (5), 367–485.

Anthérieu, S., Chesné, C., Li, R., Camus, S., Lahoz, A., Picazo, L., Turpeinen, M., Tolonen, A., Uusitalo, J., Guguen-Guillouzo, C., Guillouzo, A., 2010. Stable expression, activity, and inducibility of cytochromes P450 in differentiated HepaRG cells. Drug Metab. Dispos. 38 (3), 516–525.

Armitage, J.M., Wania, F., Arnot, J.A., 2014. Application of mass balance models and the chemical activity concept to facilitate the use of *in vitro* toxicity data for risk assessment. Environ. Sci. Technol. 48 (16), 9770–9779.

Aschauer, L., Carta, G., Vogelsang, N., Schlatter, E., Jennings, P., 2015a. Expression of xenobiotic transporters in the human renal proximal tubule cell line RPTEC/TERT1. Toxicol. In Vitro 30 (1), 95–105.

Aschauer, L., Limonciel, A., Wilmes, A., Stanzel, S., Kopp-schneider, A., Hewitt, P., Lukas, A., Leonard, M.O., Pfaller, W., Jennings, P., 2015b. Application of RPTEC/TERT1 cells for investigation of repeat dose nephrotoxicity: a transcriptomic study. Toxicol. In Vitro 30 (1), 106–116.

Aschauer, L., Gruber, L.N., Pfaller, W., Limonciel, A., Athersuch, T.J., Cavill, R., Khan, A., Gstraunthaler, G., Grillari, J., Grillari, R., Hewitt, P., Leonard, M.O., Wilmes, A., Jennings, P., 2013. Delineation of the key aspects in the regulation of epithelial monolayer formation. Mol. Cell. Biol. 33 (13), 2535–2550.

Austin, R.P., Barton, P., Mohmed, S., Riley, R.J., 2005. The binding of drugs to hepatocytes and its relationship to physicochemical properties. Drug Metab. Dispos. 33 (3), 419–425.

Avdeef, A., Box, K.J., Comer, J.E.A., Hibbert, C., Tam, K.Y., 1998. pH-Metric logP 10. Determination of liposomal membrane–water partition coefficients of ionizable drugs. Pharm. Res. 15 (2), 209–215.

Bellwon, P., Culot, M., Wilmes, A., Schmidt, T., Zurich, M.G., Schultz, L., Schmal, O., Gramowski-voss, A., Weiss, D.G., Jennings, P., Bal-Price, A., Testai, E., Dekant, W., 2015a. Cyclosporine A kinetics in brain cell cultures and its potential of crossing the blood–brain barrier. Toxicol. In Vitro 30 (1), 166–175.

Bellwon, P., Truisi, G.L., Bois, F.Y., Wilmes, A., Schmidt, T., Savary, C.C., Parmentier, C., Hewitt, P.G., Schmal, O., Josse, R., Richert, L., Guillouzo, A., Mueller, S.O., Jennings, P., Testai, E., Dekant, W., 2015b. Kinetics and dynamics of cyclosporine A in three hepatic cell culture systems. Toxicol. In Vitro 30 (1), 62–78.

Broeders, J.J.W., Blaauboer, B.J., Hermens, J.L.M., 2013. *In vitro* biokinetics of chlorpromazine and the influence of different dose metrics on effect concentrations for cytotoxicity in Balb/c 3T3, Caco-2 and HepaRG cell cultures. Toxicol. In Vitro 27 (3), 1057–1064.

Broeders, J.J.W., Blaauboer, B.J., Hermens, J.L.M., 2011. Development of a negligible depletion-solid phase microextraction method to determine the free concentration of chlorpromazine in aqueous samples containing albumin. J. Chromatogr. A 1218 (47), 8529–8535.

Broeders, J.J.W., Hermens, J.L.M., Blaauboer, B.J., Zurich, M.-G., 2015a. The in vitro biokinetics of chlorpromazine and diazepam in aggregating rat brain cell cultures after repeated exposure. Toxicol. In Vitro 30 (1), 185–191.

Broeders, J.J.W., Parmentier, C., Truisi, G.L., Jossé, R., Alexandre, E., Savary, C.C., Hewitt, P.G., Mueller, S.O., Guillouzo, A., Richert, L., Van Eijkeren, J.C.H., Hermens, J.L.M., Blaauboer, B.J., 2015b. Biokinetics of chlorpromazine in primary rat and human hepatocytes and human HepaRG cells after repeated exposure. Toxicol. In Vitro 30 (1), 52–61.

Broeders, J.J.W., Van Eijkeren, J.C.H., Blaauboer, B.J., Hermens, J.L.M., 2012. Transport of chlorpromazine in the Caco-2 cell permeability assay: a kinetic study. Chem. Res. Toxicol. 25 (7), 1442–1451.

Crean, D., Bellwon, P., Aschauer, L., Limonciel, A., Moenks, K., Hewitt, P., Schmidt, T., Herrgen, K., Dekant, W., Lukas, A., Bois, F., Wilmes, A., Jennings, P., Leonard, M.O., 2015. Development of an *in vitro* renal epithelial disease state model for xenobiotic toxicity testing. Toxicol. In Vitro 30 (1), 128–137.

Culot, M., Da Costa, A.F., Sevin, E., Szorath, E., Martinsson, S., Renftel, M., Hongmei, Y., Cecchelli, R., Lundquist, S., 2013. A simple method for assessing free brain/free

plasma ratios using an *in vitro* model of the blood brain barrier. PLoS ONE 8 (12). http://dx.doi.org/10.1371/journal.pone.0080634 e80634.
Culot, M., Lundquist, S., Vanuxeem, D., Nion, S., Landry, C., Delplace, Y., Dehouck, M.-., Berezowski, V., Fenart, L., Cecchelli, R., 2008. An *in vitro* blood–brain barrier model for high throughput (HTS) toxicological screening. Toxicol. In Vitro 22 (3), 799–811.
Debruyn, A.M.H., Gobas, F.A.P.C., 2007. The sorptive capacity of animal protein. Environ. Toxicol. Chem. 26 (9), 1803–1808.
Dörwald, F.Z., 2012. Lead Optimization for Medicinal Chemists: Pharmacokinetic Properties of Functional Groups and Organic Compounds. Wiley-VCH Verlag & Co. KGaA, Weinheim, Germany.
Endo, S., Goss, K.-U., 2011. Serum albumin binding of structurally diverse neutral organic compounds: data and models. Chem. Res. Toxicol. 24 (12), 2293–2301.
Escher, B.I., Hermens, J.L.M., 2004. Internal exposure: linking bioavailability to effects. Environ. Sci. Technol. 38 (23), 455A–462A.
Fabulas-Da Costa, A., Aijjou, R., Hachani, J., Landry, C., Cecchelli, R., Culot, M., 2013. *In vitro* blood–brain barrier model adapted to repeated-dose toxicological screening. Toxicol. In Vitro 27 (6), 1944–1953.
Groothuis, F.A., Heringa, M.B., Nicol, B., Hermens, J.L.M., Blaauboer, B.J., Kramer, N.I., 2015. Dose metric considerations in *in vitro* assays to improve quantitative *in vitro–in vivo* dose extrapolations. Toxicology 332, 30–40.
Gülden, M., Mörchel, S., Seibert, H., 2001. Factors influencing nominal effective concentrations of chemical compounds *in vitro*: cell concentration. Toxicol. In Vitro 15 (3), 233–243.
Gülden, M., Schreiner, J., Seibert, H., 2015. In vitro toxicity testing with microplate cell cultures: impact of cell binding. Toxicology 332, 41–51.
Gülden, M., Seibert, H., 2005. Impact of bioavailability on the correlation between *in vitro* cytotoxic and *in vivo* acute fish toxic concentrations of chemicals. Aquat. Toxicol. 72 (4), 327–337.
Gülden, M., Seibert, H., 2003. *In vitro–in vivo* extrapolation: estimation of human serum concentrations of chemicals equivalent to cytotoxic concentrations *in vitro*. Toxicology 189 (3), 211–222.
Hamon, J., Renner, M., Jamei, M., Lukas, A., Kopp-Schneider, A., Bois, F.Y., 2015. Quantitative in vitro to in vivo extrapolation of tissues toxicity. Toxicol. In Vitro 30 (1), 203–216.
Heringa, M.B., Hermens, J.L.M., 2003. Measurement of free concentrations using negligible depletion-solid phase microextraction (nd-SPME). TrAC, Trends Anal. Chem. 22 (9), 575–587.
Hewitt, N.J., Lechón, M.J.G., Houston, J.B., Hallifax, D., Brown, H.S., Maurel, P., Kenna, J. G., Gustavsson, L., Lohmann, C., Skonberg, C., Guillouzo, A., Tuschl, G., Li, A.P., Lecluyse, E., Groothuis, G.M.M., Hengstler, J.G., 2007. Primary hepatocytes: current understanding of the regulation of metabolic enzymes and transporter proteins, and pharmaceutical practice for the use of hepatocytes in metabolism, enzyme induction, transporter, clearance, and hepatotoxicity studies. Drug Metab. Rev. 39 (1), 159–234.
Hubatsch, I., Ragnarsson, E.G.E., Artursson, P., 2007. Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers. Nat. Protoc. 2 (9), 2111–2119.
Jossé, R., Aninat, C., Glaise, D., Dumont, J., Fessard, V., Morel, F., Poul, J.-, Guguen-Guillouzo, C., Guillouzo, A., 2008. Long-term functional stability of human HepaRG hepatocytes and use for chronic toxicity and genotoxicity studies. Drug Metab. Dispos. 36 (6), 1111–1118.
Knöbel, M., Busser, F.J.M., Rico-Rico, A., Kramer, N.I., Hermens, J.L.M., Hafner, C., Tanneberger, K., Schirmer, K., Scholz, S., 2012. Predicting adult fish acute lethality with the zebrafish embryo: relevance of test duration, endpoints, compound properties, and exposure concentration analysis. Environ. Sci. Technol. 46 (17), 9690–9700.
Kola, I., 2008. The state of innovation in drug development. Clin. Pharmacol. Ther. 83 (2), 227–230.
Kramer, N.I., Krismartina, M., Rico-Rico, A., Blaauboer, B.J., Hermens, J.L.M., 2012. Quantifying processes determining the free concentration of phenanthrene in basal cytotoxicity assays. Chem. Res. Toxicol. 25 (2), 436–445.
Kratochwil, N.A., Huber, W., Müller, F., Kansy, M., Gerber, P.R., 2004. Predicting plasma protein binding of drugs — revisited. Curr. Opin. Drug Discov. Devel. 7 (4), 507–512.
Louisse, J., De Jong, E., Van De Sandt, J.J.M., Blaauboer, B.J., Woutersen, R.A., Piersma, A.H., Rietjens, I.M.C.M., Verwei, M., 2010. The use of in vitro toxicity data and physiologically based kinetic modeling to predict dose–response curves for in vivo developmental toxicity of glycol ethers in rat and man. Toxicol. Sci. 118 (2), 470–484.
Lübberstedt, M., Müller-Vieira, U., Mayer, M., Biemel, K.M., Knöspel, F., Knobeloch, D., Nüssler, A.K., Gerlach, J.C., Zeilinger, K., 2011. HepaRG human hepatic cell line utility as a surrogate for primary human hepatocytes in drug metabolism assessment in vitro. J. Pharmacol. Toxicol. Methods 63 (1), 59–68.
Mueller, S.O., Guillouzo, A., Hewitt, P.G., Richert, L., 2015. Drug biokinetics and toxicity assessment in rat and human primary hepatocytes and HepaRG cells within the EU-funded Predict-IV project. Toxicol. In Vitro 30 (1), 19–26.
Paliwal, J.K., Smith, D.E., Cox, S.R., Berardi, R.R., Dunn-Kucharski, V.A., Elta, G.H., 1993. Stereoselective, competitive, and nonlinear plasma protein binding of ibuprofen enantiomers as determined in vivo in healthy subjects. J. Pharmacokinet. Biopharm. 21 (2), 145–161.
Park, B.K., Boobis, A., Clarke, S., Goldring, C.E.P., Jones, D., Kenna, J.G., Lambert, C., Laverty, H.G., Naisbitt, D.J., Nelson, S., Nicoll-Griffith, D.A., Obach, R.S., Routledge, P., Smith, D.A., Tweedie, D.J., Vermeulen, N., Williams, D.P., Wilson, I.D., Baillie, T. A., 2011. Managing the challenge of chemically reactive metabolites in drug development. Nat. Rev. Drug Discov. 10 (4), 292–306.
Paustenbach, D.J., 2000. The practice of exposure assessment: a state-of-the-art review. J. Toxicol. Environ. Health, Part B 3 (3), 179–291.
Pomponio, G., Savary, C.C., Parmentier, C., Bois, F., Guillouzo, A., Romanelli, L., Richert, L., Di Consiglio, E., Testai, E., 2015a. In vitro kinetics of amiodarone and its major metabolite in two human liver cell models after acute and repeated treatments. Toxicol. In Vitro 30 (1), 36–51.
Pomponio, G., Zurich, M., Schultz, L., Weiss, D.G., Romanelli, L., Gramowski-voss, A., Di Consiglio, E., Testai, E., 2015b. Amiodarone biokinetics, the formation of its major oxidative metabolite and neurotoxicity after acute and repeated exposure of brain cell cultures. Toxicol. In Vitro 30 (1), 192–202.
Ravindranath, V., Strobel, H.W., 2013. Cytochrome P450-mediated metabolism in brain: functional roles and their implications. Expert Opin. Drug Metab. Toxicol. 9 (5), 551–558.
Riedl, J., Altenburger, R., 2007. Physicochemical substance properties as indicators for unreliable exposure in microplate-based bioassays. Chemosphere 67 (11), 2210–2220.
Sasseville, V.G., Lane, J.H., Kadambi, V.J., Bouchard, P., Lee, F.W., Balani, S.K., Miwa, G. T., Smith, P.F., Alden, C.L., 2004. Testing paradigm for prediction of development-limiting barriers and human drug toxicity. Chem. Biol. Interact. 150 (1), 9–25.
Savary, C.C., Jiang, X., Aubry, M., Jossé, R., Kopp-Schneider, A., Hewitt, P., Guillouzo, A., 2015. Transcriptomic analysis of untreated and drug-treated differentiated HepaRG cells over a 2-week period. Toxicol. In Vitro 30 (1), 27–35.
Schirmer, K., Chan, A.G.J., Greenberg, B.M., Dixon, D.G., Bols, N.C., 1997. Methodology for demonstrating and measuring the photocytotoxicity of fluoranthene to fish cells in culture. Toxicol. In Vitro 11 (1-2), 107–119.
Schultz, L., Zurich, M.G., Culot, M., Da Costa, A., Landry, C., Bellwon, P., Kristl, T., Hörmann, K., Ruzek, S., Aiche, A., Reinert, K., Bielow, C., Gosselet, F., Cecchelli, R., Huber, C.G., Schroeder, O.H., Gramowski-Voss, A., Weiss, D.G., Bal-Price, A., 2015. Evaluation of drug-induced neurotoxicity based on metabolomics, proteomics and electrical activity measurements in complementary CNS in vitro models. Toxicol. In Vitro 30 (1), 138–165.
Seibert, H., Mörchel, S., Gülden, M., 2002. Factors influencing nominal effective concentrations of chemical compounds *in vitro*: medium protein concentration. Toxicol. In Vitro 16 (3), 289–297.
Seydel, J.K., Wiese, M., 2002. Drug–Membrane Interactions: Analysis, Drug Distribution, Modeling, Volume 15. Wiley-VCH Verlag GmbH & Co. KgaA, Weinheim, Germany.
Stadnicka-Michalak, J., Tanneberger, K., Schirmer, K., Ashauer, R., 2014. Measured and modeled toxicokinetics in cultured fish cells and application to *in vitro–in vivo* toxicity extrapolation. PLoS ONE 9 (3). http://dx.doi.org/10.1371/journal.pone.0092303 e92303.
Tanneberger, K., Knöbel, M., Busser, F.J.M., Sinnige, T.L., Hermens, J.L.M., Schirmer, K., 2013. Predicting fish acute toxicity using a fish gill cell line-based toxicity assay. Environ. Sci. Technol. 47 (2), 1110–1119.
Tirelli, V., Catone, T., Turco, L., Di Consiglio, E., Testai, E., De Angelis, I., 2007. Effects of the pesticide chlorpyrifos on an in vitro model of intestinal barrier. Toxicol. In Vitro 21 (2), 308–313.
Truisi, G.L., Di Consiglio, E., Parmentier, C., Savary, C.C., Pomponio, G., Bois, F., Lauer, B., Jossé, R., Hewitt, P.G., Mueller, S.O., Richert, L., Guillouzo, A., Testai, E., 2015. Understanding the biokinetics of ibuprofen after single and repeated treatments in rat and human *in vitro* liver cell systems. Toxicol. Lett. 233 (2), 172–186.
Vichi, S., Sandström Von Tobel, J., Gemma, S., Stanzel, S., Kopp-Schneider, A., Monnet-Tschudi, F., Testai, E., Zurich, M.G., 2015. Cell type-specific expression and localization of cytochrome P450 isoforms in tridimensional aggregating rat brain cell cultures. Toxicol. In Vitro 30 (1), 176–184.
Webborn, P.J.H., Parker, A.J., Denton, R.L., Riley, R.J., 2007. In vitro–in vivo extrapolation of hepatic clearance involving active uptake: theoretical and experimental aspects. Xenobiotica 37 (10-11), 1090–1109.
Wieser, M., Stadler, G., Jennings, P., Streubel, B., Pfaller, W., Ambros, P., Riedl, C., Katinger, H., Grillari, J., Grillari-Voglauer, R., 2008. hTERT alone immortalizes epithelial cells of renal proximal tubules without changing their functional characteristics. Am. J. Physiol. Ren. Physiol. 295 (5), F1365–F1375.
Wilmes, A., Bielow, C., Ranninger, C., Bellwon, P., Aschauer, L., Limonciel, A., Chassaigne, H., Kristl, T., Aiche, S., Huber, C.G., Guillou, C., Hewitt, P., Leonard, M.O., Dekant, W., Bois, F., Jennings, P., 2015. Mechanism of cisplatin proximal tubule toxicity revealed by integrating transcriptomics, proteomics, metabolomics and biokinetics. Toxicol. In Vitro 30 (1), 117–127.
Wilmes, A., Limonciel, A., Aschauer, L., Moenks, K., Bielow, C., Leonard, M.O., Hamon, J., Carpi, D., Ruzek, S., Handler, A., Schmal, O., Herrgen, K., Bellwon, P., Burek, C., Truisi, G.L., Hewitt, P., Di Consiglio, E., Testai, E., Blaauboer, B.J., Guillou, C., Huber, C.G., Lukas, A., Pfaller, W., Mueller, S.O., Bois, F.Y., Dekant, W., Jennings, P., 2013. Application of integrated transcriptomic, proteomic and metabolomic profiling for the delineation of mechanisms of drug induced cell stress. J. Proteome 79, 180–194.