Alternatives in Pharmaceutical Toxicology: Global and Focussed Approaches – Two Case Studies

Stefan O. Mueller, Gregor Tuschl and Margret Kling

Molecular Toxicology, Institute of Toxicology, Merck KGaA, Darmstadt, Germany

Summary

Safety testing of potential drugs has been and will continue to be a challenging task for the toxicologist in the pharmaceutical industry. We present two examples for the use of target-specific cell models to detect and assess species-specific toxicity. In the first example, adrenal models based on primary cells as well as a permanent human adrenal cell line were used. Both cell systems enabled a good prediction of adrenal effects in rodents, non-rodents and humans. The second example made use of primary hepatocytes. In this project, a potential drug candidate showed unexpected toxicity in vitro as well as species-specific cytochrome P450 (CYP) induction in vivo. We therefore analysed CYP induction and gene expression signatures in rat and human hepatocytes as well as in samples from in vivo animal toxicity studies. By this approach, the rat hepatocyte model correctly predicted the effects observed in rats and the in vitro / in vivo comparison enabled a solid extrapolation of consequences in humans. These examples demonstrate that an intelligent testing strategy, using alternative methods, can enable a meaningful safety assessment for humans by adding a “tailor-made” range of technologies to “classic” toxicological methods.

Keywords: hepatocytes, toxicogenomics, mechanistic models, adrenal cells

1 Introduction

Safety testing of potential drugs has been and will continue to be a challenging task for the toxicologist in the pharmaceutical industry. The “emerging technologies” such as the “omics” technologies have increased the possibilities but also the expectations to improve the efficiency of the prediction of relevant and species-specific toxicities of potential drug candidates. However, these rapid technological developments have not yet accelerated safety testing to the expected extent. Toxicology in the pharmaceutical industry is required to predict potential compound-induced toxicity in humans and also to elucidate the respective mechanisms leading to toxicity. Experiments in laboratory animals are well suited to detect toxicity in various target tissues, but the mechanisms of toxicity can be studied better in defined cellular systems. Further, these mechanistic studies in vitro can help to judge the relevance of adverse events observed in animals might have in humans. Alternative test models are therefore broadly used in the pharmaceutical industry and significant attempts have been undertaken by industry to reduce animal experimentation, thereby serving the 3R principle (Russell and Burch, 1959). In particular, alternatives to replace animal tests have been developed, e.g., hen’s egg test (HET-CAM) for irritation. Supportive analyses of mandatory in vivo experiments performed during drug development (overview of relevant guide-
lines in ICH 2005) are powerful tools to confirm and improve the interpretation and elucidation of mechanisms of observed toxicities.

One area in which alternatives are used in the pharmaceutical industry is the early characterisation of potential drug candidates prior to drug development. Here, easy to use in vitro tests are employed that comprise cytotoxicity endpoints, (e.g. trypan blue exclusion, leakage of cytotoxic enzymes, uptake of neutral red or measurement of energy expenditure), cellular stress response (e.g. activity of NF-κB and activator protein (AP)-1) and genotoxicity alerts (e.g. p53 activation and Ames assay). These assays are usually performed in established cell lines such as HepG2 or — to account for metabolic activation — in primary hepatocytes. A series of tests enables a ranking of pharmacologically active compounds. Usually, this ranking is based on in vitro safety margins, which is the difference between the toxic dose and the pharmacological dose. By these means a pre-selection of feasible drug candidates can be made that avoids useless animal experiments. An important addition to these assays is the screening for induction of cytochrome P450 mono-oxygenases (CYP), the major drug metabolising enzymes, that may cause undesired drug-drug interactions as well as toxification and/or detoxification of a drug.

A second and more sophisticated area in which new approaches are used is the so-called field of mechanistic or explanatory toxicology. One application is the analysis of samples (e.g. tissue, blood or urine) from mandatory in vivo studies for gene and protein expression, metabolic profiles or enzyme activity parameters. These analyses help to gain more insights into the mode of action of toxicities observed in vivo. Furthermore, tailored in vitro models can be used for the analysis of specific toxicity observed either in humans or animals. These studies can help to explain the toxicity, support risk assessment and allow an extrapolation of the relevance of effects observed in animals to humans.

One important field of alternative methods is hepatotoxicity. Toxicity of the liver is a major issue in pharmaceutical drug development (Ballet, 1997), and drug-induced liver injury (DILI) is the major reason for attrition in clinical studies as well as withdrawals of drugs already on the market (Fung, 2001). Unfavourable hepatic reactions are often idiosyncratic, occurring on a background of transient liver injuries in less than 1 in 10,000 patients (Kaplowitz, 2005), and this may be one reason why certain examples of human hepatotoxicity have not been predictable from standard in vitro cytotoxicity assays (Xu et al., 2004) or regulatory animal studies (Olson et al., 2000; Olson et al., 1998). Prediction of human hepatotoxicity remains poor due to strong inter-species variations in data obtained from in vivo studies and the lack of truly physiological conditions, especially the complexity of a whole organism, in in vitro experiments. However, it is worth mentioning that when in vitro assays identify a compound as a liver-toxicant, there is a more than 80% specific correlation with corresponding findings in humans (Xu et al., 2004). Several alternative models (see Fig. 1) of the in vivo situation are available to model effects related to hepatotoxicity, for example: specific enzyme action can be analysed using reconstituted CYP enzymes, cellular stress response is measured in established cell lines, CYP induction is fairly well modelled in primary hepatocytes, and drug transport can be assessed in hepatocyte sandwich culture or CACO-2 cells.

While in vivo models are used to investigate hepatic drug effects in the context of toxicokinetics and systemic influences, cell culture models provide test systems for the investigation of specific mechanisms in a precisely controlled environment (Ulrich et al., 1995). In vivo studies, limited by animal welfare/ethical concerns and difficulties to distinguish between primary and secondary toxic effects, can be supplemented and partly replaced by in vitro models to provide a more detailed understanding of the mechanism of toxicity. There are many in vivo and in vitro test systems currently in use to predict hepatotoxicity in humans. However, their application is limited due to the frequently idiosyncratic nature of liver toxicity and the inherent differences between the metabolic activity in human and non-human species. The simplicity of some in vitro systems makes it possible to specifically manipulate and analyse a small number of parameters and it allows increased throughput (see Fig. 1). However, the reduced complexity of the system translates to inherent constraints for each model. This limits their widespread use and acceptance by the regulatory authorities as an alternative to in vivo safety testing (Brandon et al., 2003).

![Fig. 1: Examples of models to analyse hepatotoxicity](image-url)
One major obstacle of some in vitro models is the limited metabolic activation of xenobiotics, mainly due to the down-regulation of CYP enzymes in cell cultures over time (Ching et al., 1996; De Smet et al., 2001). This is especially important since the phase I and phase II metabolic conversion of chemicals has great influence on their toxicity and can lead to detoxification or toxification of xenobiotics (Dahlin et al., 1984; Holme et al., 1984; Miner and Kissinger, 1979).

To overcome these problems, new and innovative strategies are being developed in order to find reliable markers that are involved not only in early toxic responses but also in chronic toxicities, both occurring at sub-lethal doses of a test compound. Furthermore, there is a strong need for a robust, long-term in vitro screening system that allows the characterisation of drug/chemical-induced toxicities after repeated dosing and helps to reduce the use of animals in toxicity testing.

Today, cultures of primary hepatocytes are used in a variety of pharmacological and toxicological experiments. For short-term experiments, hepatocytes, cultured as a monolayer on collagen film in serum-free media, are accepted as a commonly used model to reflect liver-specific metabolic activity (Richert et al., 2003; Tuschl and Mueller, 2006).

Here we describe case studies in which tailored cell models were applied to detect and assess species-specific toxicities. In the first example, adrenal cell models based on primary cells as well as a human adrenal cell line were used. Both cell systems enabled good prediction of adrenal effects in rodents, non-rodents, and humans. The second example made use of primary hepatocytes. In this project, a drug development candidate showed unexpected species-specific CYP-induction in vivo. We therefore analysed CYP-induction and potential toxicity signatures in rat and human hepatocytes as well as in samples from in vivo toxicity studies.

2 Case study 1: Models to analyse adrenal toxicity

The adrenal gland is a well-characterised target for drug-induced toxicity and one of its important features is the biosynthesis of steroids (Miller, 1995). In fact, several adrenotoxic compounds impair the synthesis of cortisol and corticosterone by enzyme inhibition (Rosol et al., 2001) and the major steroidogenic enzymes that regulate formation of cortisol and aldosterone belong to the CYP family (see Fig. 2).

Steroidogenesis is different in rodents compared to dogs and humans. Dogs and humans use the so-called Δ^4-pathway via CYP17 and produce predominantly cortisol, whereas the major glucocorticoid produced in rats is corticosterone (see Fig. 2) (Ishimura and Fujita, 1997). Since adrenal cells cannot store steroid hormones, quantification of the secreted hormones reflects the activity of steroidogenic enzymes. Analysis of the

Fig. 2: Steroidogenic pathways in the adrenals

Pregnenolone (boxed) is added to the cell culture medium to initiate steroidogenesis in H295 cells. The specific CYP 11B inhibitor metyrapone and the CYP inhibitor ketoconazole are also shown. CYP, cytochrome P450; 3ß-HSD, 3ß-hydroxysteroid dehydrogenase.
steroids secreted by adrenal cells into medium supernatants therefore indicates which steps of steroidogenesis might be affected.

2.1 Experimental Procedures

2.1.1 Chemicals

All chemicals were from Invitrogen or Sigma (Germany) unless otherwise specified.

2.1.2 Primary adrenal cell culture

Primary rat adrenal cells were isolated from male wistar rats (strain: Hsd-Cpb:WU) 8 wks-of age. Primary canine adrenal cells were isolated from a male marshall beagle (age: 4 years and 8 months). Wistar rats and the marshall beagle were obtained from Harlan-Winkelmann (Borchen, Germany).

Adrenals were dissected and collected in ice-cold wash medium. Any remaining fat and the capsule was removed from the adrenals under sterile conditions. The remaining adrenal tissue containing cortical and medulla cells was then enzymatically dissociated. The dissected adrenals were incubated in DMEM/F-12 medium containing collagenase I (2 mg/ml; Biochrom, Germany) in a shaking water-bath at 37°C for 45 minutes. To stop enzymatic dissociation, fetal calf serum was added and the cell suspension was filtered through a 70 µm cell strainer (Falcon, Germany). The filtered cell suspension was spun at 200 x g for 10 minutes and the resulting cell pellet resuspended and washed with wash medium a second time. Cells were then resuspended in phosphate-buffered saline (PBS)/0.5% bovine serum albumin and layered on a 40% Percoll gradient. The Percoll gradient was then spun at 836 x g at 4°C for 30 minutes. The upper cell layer at 40% Percoll was collected and washed twice with DMEM/F-12 medium. The adrenal cells were then resuspended in DMEM/F-12 and 0.5 x 10^5 cells were plated per well in 96-well plates. Cells were cultured in DMEM/F-12 containing 2.5 % fetal calf serum, 10 % horse serum, 0.2 % BSA and 1 µg/ml insulin at 37°C/5% CO2 in a humidified atmosphere.

2.1.3 H295 cell culture

The H295 cells are derived from a human adrenocortical carcinoma. The original cell batch was obtained from ATCC (Manassas, VA, USA; Order Number CRL-10296, Lot: 1704501) and adapted to grow as adherent monolayer.

Cells were cultured in tissue culture flasks at 37°C/5% CO2 in air in a humidified atmosphere in DMEM/F-12 supplemented with 1% ITS (6.25 µg/ml Insulin, 6.25 µg/ml transferrin; 6.25 ng/ml selenic acid, 1.25 mg/ml BSA and 5.35 mg/ml linoleic acid; BectonDickinson, Germany) and 2.5% NuSerum (BectonDickinson, Germany). Steroidogenesis was initiated by adding 10 µM pregnenolone and cells were stimulated with 300 µM cyclic adenosinemonophosphate (cAMP). For quantification of protein and steroid measurements, 4 x 10^5 cells were plated per well in 24 well plates. Cells were incubated with the test compounds for 24 h.

### Inhibition of steroidogenesis in primary rat adrenal cells

<table>
<thead>
<tr>
<th>Inhibition of</th>
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<th>Inhibition of</th>
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<tr>
<td>progesterone formation</td>
<td>corticosterone formation</td>
<td>aldosterone formation</td>
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<td>(IC50 in µM)</td>
<td>(IC50 in µM)</td>
<td>(IC50 in µM)</td>
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<tr>
<td>indicates inhibition of</td>
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<td>CYP11B1, CYP11A1</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>4</td>
<td>2</td>
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<td>Metyrapone</td>
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*Steroidogenesis was assessed as described in experimental procedures.

### Inhibition of steroidogenesis in primary canine adrenal cells

<table>
<thead>
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<th>Inhibition of</th>
<th>Inhibition of</th>
<th>Inhibition of</th>
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<tr>
<td>progesterone formation</td>
<td>aldosterone formation</td>
<td>11-desoxycortisol formation</td>
<td>cortisol formation</td>
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<tr>
<td>(IC50 in µM)</td>
<td>(IC50 in µM)</td>
<td>(IC50 in µM)</td>
<td>(IC50 in µM)</td>
</tr>
<tr>
<td>indicates inhibition of</td>
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<td>CYP11B2, CYP11A1</td>
<td>CYP17, CYP11A1</td>
</tr>
<tr>
<td>Ketoconazole</td>
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<td>4</td>
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<td>n. d.</td>
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*Steroidogenesis was assessed as described in experimental procedures.

**a** IC50 values (concentration of compound yielding half-maximal inhibition of steroid formation) were derived by non-linear curve-fitting of dose response curves using Origin Microcal (USA) software.

**b** n.d.: not determinable due to lack of activity.
2.1.4 Steroid quantification

After incubation, the cell culture medium was collected for steroid measurement and the remaining cells were lysed for quantification of protein. Aliquots of culture medium supernatants were used to quantify steroids by Jod-125 radioimmunoassays using kits for progesterone, aldosterone, corticosterone, 11-desoxycortisol and cortisol from DSL (DSL, Germany) or DPC (Biermann Diagnostica, Germany). Steroids were quantified in quadruplicate and the amount of steroid was normalized to the amount of protein per sample.

2.2 Results

In this study we used primary cultures of adrenocortical cells from rat and dog as well as a human steroidogenic cell line (Sanderson et al., 2002). The aim of the study was to determine (i) whether species-specific effects could be reproduced in vitro, (ii) which particular enzymes are affected by inhibition of steroid synthesis, and (iii) how the effects observed in animal cells compare to those in human cells.

In a first series of experiments, we analysed the effects of prototypical CYP-inhibitors in primary cultures of adrenal cells from rat and dog. The steroids progesterone, aldosterone and corticosterone (rat cells only) or cortisol and 11-desoxycortisol (canine cells only) were quantified in primary adrenal cells in order to assess the potency of test compounds to inhibit steroidogenesis. As a measure of cytotoxicity and to normalise the steroid quantification for cell numbers, we also determined the protein content of each sample. The well-characterised specific CYP11B-inhibitor metyrapone and the unspecific CYP-inhibitor ketoconazole were tested (Tab. 1 and 2).

Metyrapone induced an increase of 11-desoxycortisol and a sharp decrease of cortisol in media supernatants confirming that the activity of metyrapone as a specific CYP11B inhibitor can be followed in these models. Ketoconazole, as expected, showed no preference to inhibit a specific pathway of steroid synthesis.

In a next step, we used an established steroidogenic human adrenocortical cell line, H295 that produces cortisol as the major steroid (Sanderson et al., 2002). A similar experimental set-up was employed as described above (Tab. 3).

Again, ketoconazole and metyrapone proved their action as unspecific and CYP11B-specific CYP inhibitors, respectively. Both compounds were more potent inhibitors of steroidogenesis in H295 cells in comparison to rat or canine primary cells.

At Merck KGaA, we have used these models successfully to screen for adrenotoxic compounds. A development candidate showed adrenal toxicity (adrenal degeneration) in rat and more so in the dog. We applied the rat and canine model described above and were able to reflect the increased sensitivity of canine cells compared to rat adrenal cells. Two metabolites of the development candidate were tested also and had IC50 values of 10 to 17 μM in rat and 0.6 to 2 μM in canine cells that reflected the peak blood plasma levels of adrenotoxic doses in regulatory GLP-compliant studies in the rat and in the dog. More importantly, in human H295 cells, inhibition of steroidogenesis was apparent at 1 μM and higher for the major metabolites of the drug development candidate found in man. Since human plasma levels were less than 0.2 μM, a significant inhibition of the adrenal steroidogenesis seemed unlikely in human. In fact, no significant impairment of steroidogenesis could be detected in clinical studies performed with this drug development candidate.

In summary, the described models are therefore powerful species-specific tools to screen for potentially adrenotoxic compounds and may thus help to reduce animal testing. In addition, active doses defined in vitro can give guidance for threshold exposure levels in humans.

3 Case study 2: Models to analyse hepatic toxicities

We analysed the species-specific effects of a drug development candidate, EMD 392949 (EMD), using primary hepatocyte cultures from rat and human. EMD, a mixed peroxisome-proliferator activated receptor (PPAR) α/γ agonist, exhibited well-known "PPAR"-related toxicities in liver, muscle and thyroid (Tugwood and Montague, 2002; Willson, 2000). Furthermore, in vivo results indicated a species-specific induction of CYPs in non-rodents but not in rodents. The main questions addressed were whether CYP-induction may also be observed in human and, secondly, how EMD compares to well-characterised and marketed PPAR-drugs.

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Tab. 3: Inhibition of steroidogenesis in human adrenal H295 cells

<table>
<thead>
<tr>
<th></th>
<th>Inhibition of aldosterone formation (IC50 in μM)</th>
<th>Inhibition of 11-desoxycortisol formation (IC50 in μM)</th>
<th>Inhibition of cortisol formation (IC50 in μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CYP11B2, CYP21, CYP11A1</td>
<td>CYP11A1, CYP17, CYP21</td>
<td>CYP11B1, CYP17, CYP21, CYP11A1</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>0.41</td>
<td>10</td>
<td>0.97</td>
</tr>
<tr>
<td>Metyrapone</td>
<td>&lt; 10-2</td>
<td>n.d.</td>
<td>4.2</td>
</tr>
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</table>

* Steroidogenesis was assessed as described in experimental procedures.

* IC50 values (concentration of compound yielding half-maximal inhibition of steroid formation) were derived by non-linear curve-fitting of dose response curves using Origin Microcal (USA) software.

* IC50 of metyrapone could not be determined, since at all concentrations tested (10-1000 μM) aldosterone formation was inhibited by more than 80%. The IC50 is therefore given as less than the lowest concentration tested.

* n.d.: not determinable due to lack of activity.
Global gene-expression profiles using rat whole genome chips (Affymetrix, USA) were generated. A comparison of global gene-expression profiling in the liver of rats treated for 17 weeks with EMD to that seen in rat hepatocytes treated for 24 h with 3, 30 and 100 μM EMD revealed minimal overlap. The same samples were then subjected to a focussed analysis of selected marker genes, including well-known PPAR-target genes and CYPs, using TaqMan™ low density arrays (TLDA; Applied Biosystems, Germany). This analysis revealed a near perfect match of the alterations in the expression of selected PPAR-target and CYP genes between the liver samples from the sub-chronic rat toxicity study and the expression profile determined in primary rat hepatocyte cultures (Fig. 3a and b). These results proved that for the selected subset of markers, primary hepatocytes are predictive of effects observed in long-term in vivo studies.

We then compared EMD with Fenofibrate, a PPARα agonist, Muraglitazar, a mixed PPARα/γ agonist and Pioglitazone, a PPARγ agonist (Harrity et al., 2006; Willson, 2000). Compound specific gene expression changes were examined in primary rat and human hepatocytes (Tuschi et al., 2007; Fig. 3b and c). EMD caused strong induction of CYP 2C and 3A, the major drug metabolizing enzymes, in human hepatocytes but not rat hepatocytes, indicating that EMD is a human CYP inducer. Furthermore, EMD was very similar to the typical PPARα-agonist Fenofibrate for the selected genes (e.g. induction of genes involved in fatty acid oxidation; see Fig. 3) and also on a global level using whole genome chips (data not shown). The results for the human hepatocytes showed a large variability of the three different human hepatocyte preparations analysed, indicating the necessity to analyse cells from several human donors in order to get reliable results.

Overall, EMD was comparable to the PPARα agonist Fenofibrate but less so to the mixed PPARα/γ agonist Muraglitazar or the selective PPARγ agonist Pioglitazone. This study showed that in vitro models could have high predictive power for effects observed in vivo and—
more importantly – allowed an extrapolation of potential effects in humans.

4 Conclusions

The two examples given here show that in vitro models can be powerful alternatives to animal experiments. In the first example, in vitro systems have been used to screen compounds for adrenergic effects and to elucidate mode of actions observed in vivo. In addition, these assays help in the pre-selection of drug development candidates to avoid futile animal experiments. Furthermore, in vitro results give also indications for threshold levels in humans, thereby improving human risk assessment. The approaches described exemplify how mandatory in vivo studies can be improved by extracting a maximum of information from one experiment. This clearly improves the predictivity of animal experimentation; and, in addition, reduces the number of animals required for further studies. By comparing effects observed in vitro and in vivo, a relevant extrapolation of potential effects in humans is possible. To conclude, these examples demonstrate that an intelligent testing strategy, using alternative methods, can enable a meaningful safety assessment for humans by adding a “tailormade” range of technologies to “classic” toxicological methods.

References


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Correspondence to
PD Dr. Stefan O. Mueller
Molecular Toxicology
Institute of Toxicology, Merck KGaA
Frankfurter Str. 250
D-64293 Darmstadt
Germany
stefan.o.mueller@merck.de