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IN VITRO SYSTEMS

Pharmacokinetics explain in vivo/in vitro discrepancies of carcinogen-induced gene expression alterations in rat liver and cultivated hepatocytes

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Abstract Cultivated hepatocytes represent a well-established in vitro system. However, the applicability of hepatocytes in toxicogenomics is still controversially discussed. Recently, an in vivo/in vitro discrepancy has been described, whereby the non-genotoxic rat liver carcinogen methapyrilene alters the expression of the metabolizing genes SULT1A1 and ABAT, as well as the DNA damage response gene GADD34 in vitro, but not in vivo. If the collagen sandwich cultures of hepatocytes really produce false-positive data, this would compromise its application in toxicogenomics. To revisit the putative in vivo/in vitro discrepancy, we first analyzed and modeled methapyrilene concentrations in the portal vein of rats. The relatively short half-life of 2.8 h implies a rapid decrease in orally administered methapyrilene in vivo below concentrations that can cause gene expression alterations. This corresponded to the time-dependent alteration levels of GADD34, ABAT and SULT1A1 RNA in the liver: RNA levels are altered 1, 6 and 12 h after methapyrilene administration, but return to control levels after 24 and 72 h. In contrast, methapyrilene concentrations in the culture medium supernatant of primary rat hepatocyte cultures decreased slowly. This explains why GADD34, ABAT and SULT1A1 were still deregulated after 24 h exposure in vitro, but not in vivo. It should also be considered that the earliest analyzed time point in the previous in vivo studies was 24 h after methapyrilene administration. In conclusion, previously observed in vitro/in vivo discrepancy can be explained by different pharmacokinetics present in vitro and in vivo. When the in vivo half-life is short, levels of some initially altered genes may have returned to control levels already 24 h after administration.

Keywords Hepatocyte in vitro system · Alternative methods · In vitro/in vivo comparison · Genotoxic and non-genotoxic carcinogens · In vitro pharmacokinetics

Abbreviations

SULT1A1	Sulfotransferase
ABAT	4-aminobutyrate aminotransferase
GADD34	Growth arrest and DNA damage inducible gene 34
ABCB1	Multi drug resistance protein 1NQO1: NADPH-quinone oxidoreductase

Introduction

Cultivated primary hepatocytes represent a well-established in vitro system for the study of drug metabolism and

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enzyme induction (Hewitt et al. 2007; Knobeloch et al. 2012; Bauer et al. 2009; Ullrich et al. 2009; Boess et al. 2003; Doktorova et al. 2012; Hengstler et al. 2000; Vinken et al. 2011). In recent years, cultivated hepatocytes have also been applied in gene expression studies aimed at the identification of hepatotoxic or carcinogenic compounds (Mathijs et al. 2010 2010; Hrach et al. 2011; van Kesteren et al. 2011). These studies were prompted by previous in vivo experiments demonstrating that genotoxic and non-genotoxic liver carcinogens can be identified by test compound-induced pattern of gene expression in the livers of rats (Ellinger-Ziegelbauer et al. 2005, 2008; Fielden et al. 2007; Uehara et al. 2011).

However, there are two major limitations when using cultivated primary hepatocytes in toxicogenomics. Firstly, hepatocytes undergo massive gene expression alterations, particularly during the first 24 h in culture (Godoy et al. 2009, 2010a, b; Zellmer et al. 2010). Therefore, gene expression alterations induced by test compounds have to be analyzed against a "noisy" background. Secondly, huge discrepancies between test compound-induced gene expression alterations in the liver in vivo and in hepatocytes in vitro have been reported. When rats were treated with 1.5 g/kg paracetamol, 1,349 genes were significantly up- or down-regulated (Kienhuis et al. 2009). Surprisingly, there was almost no overlap with gene expression alterations induced in cultivated hepatocytes. When rat hepatocytes were cultivated in sandwich culture using a standard hepatocyte medium, 368 genes were up- or down-regulated after incubation with paracetamol. Only two of the 368 in vitro deregulated genes overlapped with the 1,349 genes altered in vivo (Kienhuis et al. 2009). The in vivo/in vitro overlap was not substantially improved by adding the liver enzyme inducers phenobarbital, dexamethasone and beta-naphthoflavone to the hepatocyte culture medium (Kienhuis et al. 2009). The limitation of this study (Kienhuis et al. 2009) was that Wistar rats were used for the in vitro studies, whereas F344/N rats were used for the in vivo experiments. Nevertheless, it seems unlikely that the huge in vivo/in vitro discrepancy can be explained only by the difference between the two rat strains.

An even more severe in vivo/in vitro discrepancy can be observed by comparing the in vitro experiments of Beekmann et al. (2006) to the in vivo study of Ellinger-Ziegelbauer et al. (2008). In an inter-laboratory study by Beekmann et al. (2006), four independent laboratories observed that the non-genotoxic rat liver carcinogen methapyrilene increased RNA levels of the DNA damage response gene protein phosphatase 1 (GADD34) and decreased the expression of the metabolizing enzymes sulfotransferase 1A1 (SULT1A1) and 4-aminobutyrate aminotransferase (ABAT) in cultivated rat hepatocytes. This was later confirmed by our group (Schug et al. 2008; Heise et al. 2012). However, in the in vivo study of Ellinger-Ziegelbauer et al. (2005, 2008), methapyrilene did not significantly alter the expressions of GADD34, SULT1A1 and ABAT in livers of rats for all tested doses and time points. If this in vivo/in vitro discrepancy is true, this suggests that the in vitro system with cultivated hepatocytes produces false-positive data. Under such circumstances, further investment into in vitro gene expression profiling studies aimed at classifying different classes of hepatotoxic compounds would be unnecessary.

The high relevance of hepatocyte in vitro systems, for example, in the large cooperative European Collaborative Research Project SEURAT-1, required that we revisit the case of methapyrilene, which to our knowledge represents one of the best documented in vitro/in vivo discrepancies. We report that although all data (Beekmann et al. 2006; Ellinger-Ziegelbauer et al. 2005, 2008) are reproducible, the putative discrepancy can be explained by differences in the underlying mechanism of both systems. Furthermore, our current study shows that the in vitro system realistically reflects the in vivo situation.

Materials and methods

Cell culture materials and chemicals

Williams Medium E, Penicillin/Streptomycin solution and SeraPlus (FCS) were purchased from PAN-Biotech (Aidenbach, Germany). Gentamicin (10 mg/mL) was purchased from Invitrogen Corp. (Karlsruhe, Germany). Dexamethasone and methapyrilene were ordered from Sigma–Aldrich Chemie GmbH (Taufkirchen, Germany). Rat-tail tendon collagen I for sandwich culture was provided by Roche (Mannheim, Germany) and 10× DMEM was ordered from Biozol (Eching, Germany).

Animals

Male Wistar rats with a body weight of 220–300 g were purchased from Charles River (Sulzfeld, Germany). The animals had free access to food (sniff, Soest, Germany) and water and were kept under controlled temperature (18–26 °C), humidity (30–70 %) and lighting (12 h light/dark circle). Before using, the animals were acclimated for a minimum of 6 days. This study was approved by the local committee for the welfare of experimental animals and was performed in accordance with national legislation.

Isolation and cultivation of hepatocytes

Primary hepatocytes were isolated from male Wistar rats using a modified two-step isolation method described by

Hengstler et al. (2000): The animals were anesthetized with an i.p. injection of a mixture of 20 mg/kg body weight xylazine (Rompun 2 %, Bayer, Leverkusen, Germany) and 120 mg/kg body weight ketamine (Ratiopharm, Ulm, Germany). The liver perfusion was performed via the vena portae for 15 min at 37 °C with an EGTA-containing buffer. Constant temperature was achieved using an inline heating system (SAHARAInline, Transmed Sarstedt Group, Bad Wünnenberg, Germany). The EGTA buffer consisted of 248 mL glucose solution (9 g/L D-glucose), 40 mL KH buffer (60 g/L NaCl, 1.75 g/L KCl and 1.6 g/L KH_2PO_4; adjusted to pH 7.4), 40 mL HEPES buffer (60 g/L HEPES; adjusted to pH 8.5), 60 mL amino acid solution (0.27 g/L L-alanine, 0.14 g/L L-aspartic acid, 0.4 g/L L-asparagine, 0.27 g/L L-citrulline, 0.14 g/L L-cysteine, 1 g/L L-histidine, 1 g/L L-glutamic acid, 1 g/L L-glycine, 0.4 g/L L-isoleucine, 0.8 g/L L-leucine, 1.3 g/L L-lysine, 0.55 g/L L-methionine, 0.65 g/L L-ornithine, 0.55 g/L L-phenylalanine, 0.55 g/L L-proline, 0.65 g/L L-serine, 1.35 g/L L-threonine, 0.65 g/L L-tryptophan, 0.55 g/L L-tyrosine, 0.8 g/L L-valine; amino acids that could not be dissolved at neutral pH were dissolved by addition of 10 N NaOH at pH 11.0 and afterward adjusted to pH 7.6), 2 mL glutamine solution (7 g/L L-glutamine, freshly prepared) and 0.8 mL EGTA solution (47.5 g/L EGTA, dissolved by addition of NaOH, adjusted to pH 7.6). Subsequently, perfusion was continued for 15 min with prewarmed collagenase buffer (37 °C) consisting of 155 mL glucose solution, 25 mL KH buffer, 25 mL HEPES buffer, 38 mL amino acid solution, 10 mL $CaCl_2$ solution (19 g/L $CaCl_2 \times 2\ H_2O$), 2.5 mL glutamine solution and 80 mg collagenase type I (Sigma, Taufkirchen, Germany). After perfusion, the liver was dissected and dissociated in suspension buffer (124 mL glucose solution, 20 mL KH buffer, 20 mL HEPES buffer (pH 7.6), 30 mL amino acid solution, 2 mL glutamine solution, 1.6 mL $CaCl_2$ solution, 0.8 mL $MgSO_4$ solution (24.6 g/L $MgSO_4 \times 7\ H_2O$) and 0.4 g bovine serum albumin). The liver cell suspension was filtered through a 100-µm cell strainer, centrifuged for 5 min at $50 \times g$, washed twice with suspension buffer, centrifuged again and resuspended in 30 mL suspension buffer. Cell viability was determined by Trypan blue exclusion rate, and only hepatocytes with viability greater than 80 % were used. The collagen sandwich cultures were prepared by adding 12 mL of 0.2 % (v/v) acetic acid to 10 mg lyophilized collagen. The collagen was dissolved over night at 4 °C, 1.2 mL of 10× DMEM was added, and the acid solution was neutralized by adding 1 M NaOH solution. For each well of the 6-well plate (Sarstedt, Nümbrecht, Germany), 250 µL of the collagen solution was added and solidified for 30 min. The cells were seeded at a density of 1×10^6 cells per well and for attachment, 2 mL of Williams Medium E (WME) (with 10 % FCS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 10 µg/mL gentamicin, 100 nM dexamethasone) was added. Cells were cultured at 37 °C and 5 % CO_2 in a humidified atmosphere. Three hour after seeding, cells that attached to the first layer of the collagen sandwich were washed with warm (37 °C) WME. The medium was removed again and a second layer of collagen was added. After 30 min of gelation, WME was added, including the same additives mentioned before, but without FCS. The cells were incubated over night before methapyrilene treatment.

Methapyrilene in vitro study

After incubation over night, the sandwich cultures were exposed to 100 µM methapyrilene hydrochloride (HCl) in WME (100 U/mL penicillin, 0.1 mg/mL streptomycin, 10 µg/mL gentamicin, 100 nM dexamethasone) for 24 h. At the end of the exposure, the cells were harvested and RNA was collected. For each condition, samples from three technical replicates of one experiment were harvested. The methapyrilene concentrations correspond to Heise et al. (2012).

RNA isolation and processing from primary rat hepatocytes in culture

After the treatment period, the medium was removed and 1 mL of QIAzol (QIAGEN, Hilden, Germany) was added immediately. RNA isolation was performed according to the manufacturer's instructions. The RNA was then reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit from Applied Biosystems (Darmstadt, Germany).

Rat in vivo study

Twenty-four male Wistar rats, with 220–300 g body weight, were fasted for 4 h, but were given water ad libitum, before receiving a single oral dose of 60 mg methapyrilene HCl per kg of body weight. The exposure in vivo corresponds to Ellinger-Ziegelbauer et al. (2005). Six time points and three biological replicates per time point were chosen for sample collection: 0 h (untreated animals) 1, 6, 12, 24 and 72 h after methapyrilene HCl administration and two further control time points at 6 and 12 h after the animals were gavaged with the solvent control (water) only. After the appropriate time, the rats were killed with CO_2 and the liver was excised immediately. A piece of the front area of the right liver lobe was resected for RNA collection, placed in a vial with 3–4 mL QIAzol and homogenized by ultra turrax technology. RNA isolation was performed according to the manufacturer's instructions. Subsequently, the RNA was reverse transcribed into

cDNA with the High Capacity cDNA Reverse Transcription Kit from Applied Biosystems (Darmstadt, Germany).

Gene expression analysis

Quantitative real-time RT-PCR with TaqMan probes were used for gene expression analysis. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was chosen as the endogenous control (Applied Biosystems, Darmstadt, Germany). The expression assays applied were as follows: Rn01749022_g1 (*GAPDH*), Rn00578656_m1 (*ABAT*), Rn00591894_m1 (*GADD34*), Rn01510633_m1 *SULT1A1*), Rn00561753_m1 (*ABCB1*), Rn00566528_m1 (*NQO1*) (Applied Biosystems, Darmstadt, Germany); 100 ng of cDNA was used per reaction, and the PCR conditions were according to the standard specifications recommended by Applied Biosystems. The $2^{-\Delta\Delta CT}$ method was used for the calculation of the relative expression, and the threshold was manually set to 0.2. Freshly isolated, untreated hepatocytes were set as the calibrator, and time matched, untreated cells were used as controls.

Analysis of methapyrilene

Primary rat hepatocytes were isolated and plated at a density of $1x10^6$ cells per well in 6-well plate format collagen sandwich condition as mentioned above. After incubation over night, the sandwich cultures were exposed to 100 µM methapyrilene HCl (from an aqueous stock solution) in 3 mL WME (100 U/mL penicillin, 0.1 mg/mL streptomycin, 10 µg/mL gentamicin, 100 nM dexamethasone) for 0, 1, 6, 12, 24 and 72 h. After the appropriate incubation time, the medium was collected and frozen at −20 °C until all samples were collected. Subsequently, the methapyrilene content in the medium was analyzed by reversed phase high-performance liquid chromatography (HPLC). The HPLC system contained an HPLC pump from Shimadzu LC10AD, a flow controller (Shimadzu SCL 10Avp), a fluorimetric detector (Shimadzu RF 10-AXL), an autosampler (Shimadzu SIL10A) and an LC solution integrator from Shimadzu. For the mobile phase, a solution of 20 % acetonitrile, 3 % tetrahydrofuran and 77 % 25 mM $NH_4H_2PO_4$ buffer pH 3.5 were applied. The cell supernatants were diluted with mobile phase solution in a 1:2 ratio and centrifuged at 16,100×*g* for 2 min; 20 µL of the supernatant was automatically injected into a Nucleodur 100-5 C18 column (Macherey and Nagel, 125 × 3 mm). The run was performed with a pressure of 180 bars and a flow rate of 0.8 mL/min. After a retention time of 4.5 min, the compound was detected with a fluorimetric detector at an excitation wave length of 310 nm and an emission wave length of 360 nm. To determine the methapyrilene HCl concentration in the cell supernatants, an initial calibration measurement was performed for methapyrilene HCl standard solutions of various concentrations. The obtained peak areas were used to generate a calibration function, and its slope, intercept and coefficient of determination were calculated. The methapyrilene HCl concentrations of the samples were calculated from the slope of the calibration function.

Pharmacokinetic modeling

Briefly, a physiologically based rat model was set up with seven separate tissues plus the portal vein. Rat physiological parameters were taken from the literature (Brown et al. 1997). The tissue:blood partition coefficients were calculated according to Schmitt (2008). Metabolism in rats was modeled using the published data (Kelly et al. 1990). Administration of methapyrilene HCl by gavage was modeled as a single dose with an absorption half-life of 0.6 h. The extent of absorption was set at 4 % (Calandre et al. 1981). Further details are given in Mielke et al. (2010).

Statistical analysis

Global differences between mRNA levels from control and methapyrilene HCl-treated animals were checked using the *F* test. Pairwise comparisons adjusting for multiple testing were performed using the Dunnett test. In case of ABAT, SULT1A1 and ABCB1, the three control groups showed no significant difference according to the *F* test. These groups were used as the control group for the Dunnett test. In case of NQO1 and GADD34, the *F* test detected differences between the three control time points. Here, the observations at the first time point (0 h, untreated) were used as controls for the Dunnett test.

For the in vitro data, we applied one-sided *t* tests for paired samples assuming that for GADD34 the treated cells showed higher expression than the untreated controls and vice versa for ABAT and SULT1A1.

All tests were performed at a level $\alpha = 0.05$. All calculations and tests were performed using SPSS, version 20.

Results

Incubation of cultivated rat hepatocytes with 100 µM methapyrilene HCl for 24 h caused a significant decrease in mRNA levels encoding the metabolizing enzymes ABAT and SULT1A1 and increased the mRNA level encoding the DNA damage response gene GADD34 (Fig. 1). The result confirms the data of Beekmann et al. (2006) and corresponds to Schug et al. (2008). Similar effects as with the already cytotoxic methapyrilene

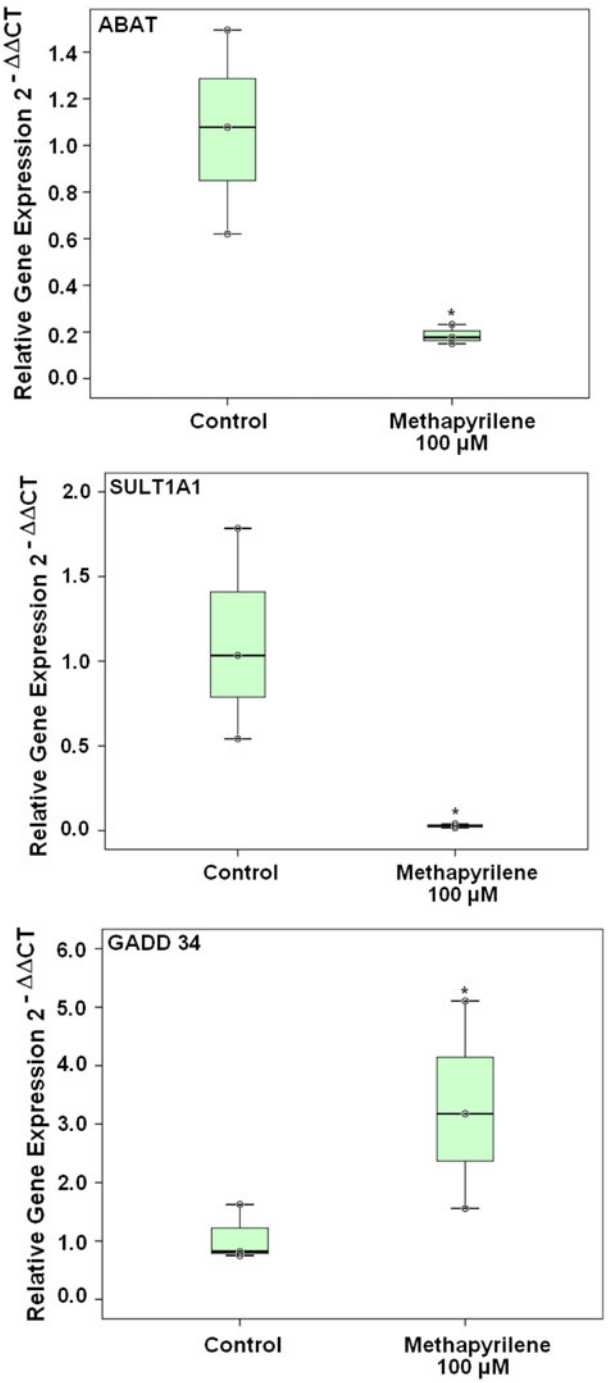
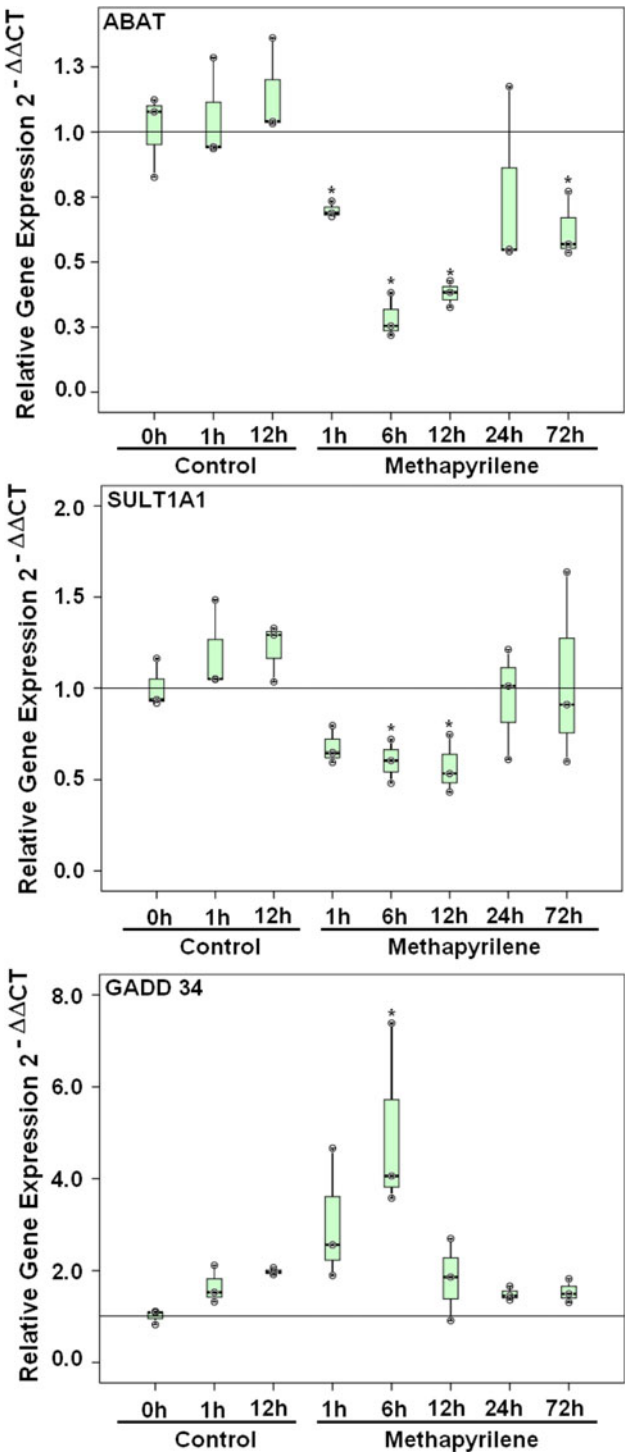


Fig. 1 Influence of methapyrilene on RNA levels of ABAT, SULT1A1 and GADD34 in cultivated rat hepatocytes. Incubations were performed with 100 µM methapyrilene for 24 h. Methapyrilene suppresses ABAT and SULT1A1 and induces GADD34 expression. Data were obtained from three technical replicates of one experiment. An expression level of 1 corresponds to RNA levels at the beginning of the respective incubation periods. The *horizontal line* in the *middle* of each *boxplot* represents the median. The *edges* of a *box mark* the 25th and the 75th percentiles. The *whiskers* show the range of values that fall within 1.5 *box* length. The data of the three technical replicates are visualized by *black circles*. One-sided *t* tests for paired samples showed significant differences between control and exposed samples at a level $\alpha < 0.05$

concentration of 100 µM have been reported for lower, non-cytotoxic concentrations (Heise et al. 2012). To compare the in vitro data to the in vivo situation, rats received 60 mg/kg body weight methapyrilene HCl by gavage. Rats were sacrificed 1, 6, 12, 24 and 72 h after administration of the test compound. ABAT and SULT1A1 expression showed a time-dependent decrease compared to the in vitro situation with the largest decrease observed after 6 and 12 h. After 24 h and 3 days, expression of these two genes increased to control levels (Fig. 2). In contrast, GADD34 mRNA expression increased 1 and 6 h after administration and returned to control levels after 12, 24 and 72 h (Fig. 2). To control for possible artefacts caused by circadian expression alterations, solvent controls were not only included at the beginning (0 h) but also 1 and 12 h after compound administration (Fig. 2). No major circadian variations were observed for the three controls for ABAT, SULT1A1 and GADD34 mRNAs.

To obtain an overview of concentrations of methapyrilene HCl in the portal vein after the administration of 60 mg/kg by gavage, a recently published physiologically based toxicokinetic modeling approach was used (Mielke et al. 2010). According to the simulation, the peak concentration in the portal vein was 1.3 µM and decreased to 1.07, 0.14, 0.077, 0.03 and 0.0009 µM after 1, 6, 12, 24 and 72 h, respectively (Fig. 3a). In contrast, the decrease in methapyrilene HCl concentrations in vitro was much slower (Fig. 3b). After incubation periods of 24 and 72 h, the concentration of methapyrilene HCl was still above 10 µM in the medium, only a tenfold decrease from the initial concentration added to the hepatocytes.

Having analyzed three genes (ABAT, SULT1A and GADD34) that were not significantly altered in the study of Ellinger-Ziegelbauer et al. (2005, 2008), we next focused on two genes that were significantly increased: The drug transporter, ABCB1, was strongly upregulated (22.8-fold) 24 h after methapyrilene HCl administration (Ellinger-Ziegelbauer et al. 2005). In contrast, the mRNA for plasma membrane oxidoreductase NQO1 was only weakly elevated (1.76-fold; Ellinger-Ziegelbauer et al. 2005). Methapyrilene HCl also induced the expression of both genes in the current study (Fig. 4). ABCB1 mRNA increased after 6 h, with highest expression after 12 h, and was still above control levels 24 and 72 h after administration of methapyrilene HCl (Fig. 4a) to rats in vivo. Similarly, NQO1 mRNA was still increased 24 h after administration (Fig. 4b). Therefore, ABCB1 and NQO1 mRNAs differ from GADD34, ABAT and SULT1A1 mRNAs because their return to control levels requires a longer time period. mRNA levels encoding these two genes were previously increased by methapyrilene in vitro also (Ellinger-Ziegelbauer et al. 2005).



◀ **Fig. 2** Influence of methapyrilene on ABAT, SULT1A1 and GADD34 RNA levels in rat livers in vivo. ABAT and SULT1A1 are suppressed only transiently but return to control levels within 24 h. GADD34 is transiently induced but no longer distinguishable from controls 24 h after methapyrilene administration. To control for possible circadian expression alterations, additional solvent controls were analyzed 1 and 12 h after compound administration besides immediately killing the rats (0 h). Data were obtained from three rats per time point. An expression level of one corresponds to gene expression levels of livers from untreated control animals (0 h). The median is shown as a *horizontal line* in the *middle* of each *boxplot*. The *edges* of a *box mark* the 25th and the 75th percentiles. The *whiskers* show the range of values that fall within 1.5 *box* length. The data of the three biological replicates are visualized by *black circles*. According to the *F* test, differences between control and exposed samples can be observed at a level $\alpha < 0.05$. *denotes *p* values below 0.05

Discussion

In this study, we revisited a well-documented in vivo/in vitro discrepancy where methapyrilene has been shown to cause gene expression alterations in cultivated rat hepatocytes, but not in rat liver in vivo (Beekmann et al. 2006; Schug et al. 2008; Ellinger-Ziegelbauer et al. 2005, 2008). This is a critical observation, because if the in vitro system really induces false-positive results, further investment into its development as a tool for toxicogenomics for the in vivo situation is not justified. However, the present data demonstrate that the putative discrepancy has a pharmacokinetic explanation.

In the in vivo study (Ellinger-Ziegelbauer et al. 2008), methapyrilene HCl was administered to rats by gavage, and liver tissue was analyzed 24 h later. A previous in vitro study using the same methods presented here (Schug et al. 2008) showed that 0.02 μM methapyrilene HCl had no influence on ABAT, SULT1A1 and GADD34 expression; 0.39 μM methapyrilene HCl had a slight effect on ABAT and SULT1A1 but none on GADD34; and 6.25 μM altered expression levels of all three genes after 24 h exposure. The physiologically based rat model applied in the present study demonstrated that 1 h after oral administration of methapyrilene HCl, the concentration in the portal vein was 1.07 μM, but decreased to 0.077 and 0.03 μM after 12 and 24 h, respectively. Therefore, it can be expected that after 12 or 24 h, at the latest, methapyrilene HCl concentrations were below levels that could influence the expression of the analyzed genes. The return of RNA concentrations to control levels, therefore, depends on the RNA half-life, in the case of increased gene expression (GADD34), and on the recovery of the expression machinery, as is the case for suppressed RNA levels (ABAT and SULT1A1). The present data show that all three genes (GADD34, ABAT and SULT1A1) returned to control levels 24 h after administration of methapyrilene HCl in rats. This is a plausible explanation as to why GADD34, ABAT and SULT1A1 were not shown to be altered in previous studies (Ellinger-Ziegelbauer et al. 2005, 2008) where the earliest analyzed time point was 24 h after the administration of methapyrilene HCl.

In vitro, the decrease in methapyrilene concentration was much slower compared to the in vivo situation (Fig. 3b). Immediately after pipetting the stock solution of

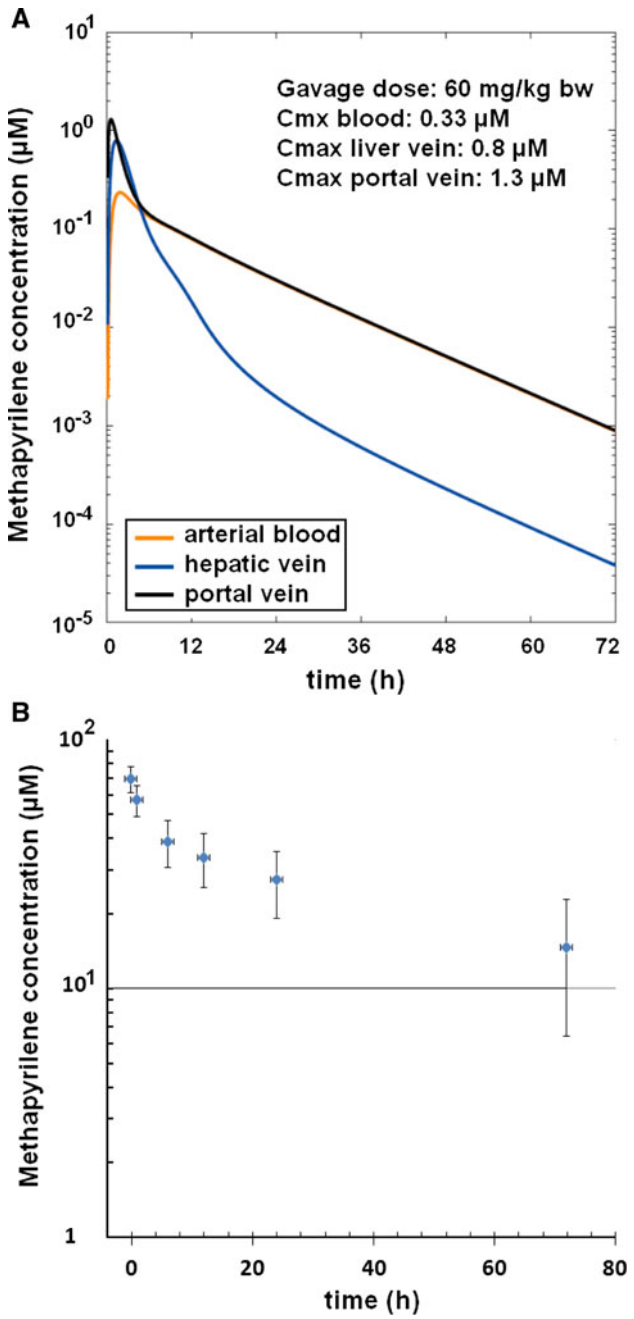


Fig. 3 **a** Simulated concentration–time profile of methapyrilene in the portal vein after oral administration of 60 mg/kg body weight obtained from a physiologically based rat model. The *peak* concentration in the portal vein was 1.3 µM. 1.07, 0.14, 0.077, 0.030 and 0.0009 µM methapyrilene were simulated in the portal vein 1, 6, 12, 24 and 72 h after administration. **b** Concentration time profile of methapyrilene in the culture medium supernatant of rat hepatocyte cultures. Data were obtained from three technical replicates of one donor animal. The data points represent mean values from the appropriate replicates together with the SE

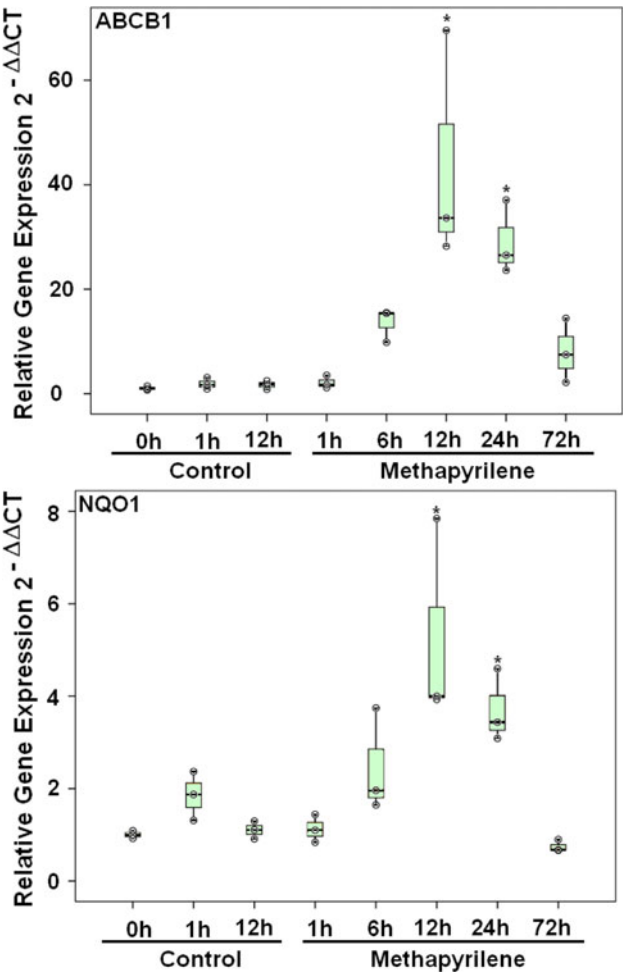


Fig. 4 Influence of methapyrilene on RNA levels of ABCB1 and NQO1 in rat livers. The same liver tissue as used for the analysis of ABAT, SULT1A1 and GADD34 in Fig. 2 was analyzed. A longer recovery period is required until ABCB1 and NQO1 return to control levels compared to the genes shown in Fig. 2. The experimental design is the same as described in Fig. 2. According to the *F* test, differences between control and exposed samples can be observed at a level $\alpha < 0.05$. *denotes *p* values < 0.05

methapyrilene HCl to the sandwich culture, a decrease below the theoretical concentration of 100 µM was measured. One possible explanation is the adsorption of the test compound to the collagen gel and to the plastic dish. However, even after incubation periods of 24 and 72 h, methapyrilene HCl was still clearly above 10 µM, concentrations that are sufficient to influence the expression levels of ABAT, SULT1A1 or GADD34. The observed difference is plausible, since the number of hepatocytes is approximately 1 million hepatocytes in the cell culture, whereas in vivo, 600–800 million hepatocytes (the average hepatocyte numbers of adult rat livers of 250 g) are present. Because the number of hepatocytes determine the amount of metabolizing hepatic enzymes, the internal clearance ($Cl_{int} = V_{max}/K_m$) is 600–800-fold higher in vivo as compared to the in vitro situation. The different pharmacokinetics in the 6-well dish compared to the in vivo situation is an interesting example to demonstrate the need

for so-called in vitro biokinetic studies, in particular, if effects are to be monitored over a time period of several days (Pelkonen et al. 2008). We think that the different pharmacokinetics of methapyrilene HCl in vivo and in vitro sufficiently explains the previously reported discrepancy.

In contrast to ABAT, SULT1A and GADD34, no in vivo/in vitro discrepancy was observed for ABCB1 and NQO1. Both ABCB1 and NQO1 are induced by methapyrilene in vivo (Ellinger-Ziegelbauer et al. 2005, 2008) and in rat hepatocytes in vitro (Heise et al. 2012). This may be explained by a longer half-life of ABCB1 and NQO1 mRNA compared to GADD34 RNA (Figs. 2, 3) or by a longer lasting activation of the corresponding transcription machinery.

When in vivo studies are performed using compounds with relatively short half-lives, one should be aware that only RNA species with either relatively long recovery periods (for downregulated genes) or with relatively long half-lives (for induced genes) are captured if the first time point is analyzed 24 h after compound administration (24 h sampling periods are usually preferred in in vivo studies to avoid artifacts by circadian expression alterations). However, because of the redundancy of individual RNA species (with respect to constructing classification algorithms), these "stable RNA species" appear according to the already performed analysis in vivo still sufficient to identify the relevant pathways.

In conclusion, we have shown that a previously reported in vivo/in vitro discrepancy can be explained by the different pharmacokinetics in both systems. The discrepancy does not seem to be a consequence of qualitative differences between the in vitro and in vivo systems.

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Conflict of interest The authors declare that they have no conflict of interest.

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