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Title page

Comparison of coumarin-induced toxicity between sandwich-cultured primary rat hepatocytes and rats *in vivo*: a toxicogenomics approach

Anne S. Kienhuis, Heleen M. Wortelboer, Jean-Christophe Hoflack, Edwin J. Moonen, Jos C.S. Kleinjans, Ben van Ommen, Joost H.M. van Delft, and Rob H. Stierum

Business Unit Biosciences, TNO Quality of Life, Zeist, the Netherlands (A.S.K., H.M.W., B.v.O., R.H.S.); Department of Health Risk Analysis and Toxicology, University of Maastricht, Maastricht, the Netherlands (A.S.K., E.J.M., J.C.S.K., J.H.M.v.D.); Independent author (J.C.H.)

Running title page

Coumarin-induced hepatotoxicity in rats *in vitro* and *in vivo*

Corresponding author

Rob H. Stierum, TNO Quality of Life, Business Unit Biosciences, Physiological Genomics, P.O. Box 360 (PP 8), 3700 AJ, Zeist, The Netherlands. Telephone: +31 30 694 4545. Telefax: +31 30 696 0264. E-mail: rob.stierum@voeding.tno.nl.

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List of abbreviations

CYP450, cytochrome P450; PB, phenobarbital; DEX, dexamethasone; β-NF, β-naphthoflavone; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PBS, phosphate buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; i.p., intraperitoneally; ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; LDH, lactate dehydrogenase; ALP, alkaline phosphatase; GGT, γ-glutamyl transferase; DMSO, dimethylsulphoxide; *o*-HPAA, *o*-hydroxyphenylacetic acid; TIGR, The Institute for Genomic Research; SSC, sodium chloride sodium citrate; BRB, Biometric Research Branch; DAVID, Database for Annotation, Visualization and Integrated Discovery; KEGG, Kyoto Encyclopedia of Genes and Genomes; PCA, principal component analysis; CE, coumarin 3,4-epoxide; *o*-HPA, *o*-hydroxyphenylacetaldehyde.

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Abstract

Sandwich-cultured primary rat hepatocytes are often used as an *in vitro* model in toxicology and pharmacology. Loss of liver specific functions, in particular the decline of cytochrome P450 (CYP450) enzyme activity, however, limits the value of this model for prediction of *in vivo* toxicity. In this study, we investigated whether a hepatic *in vitro* system with improved metabolic competence enhances the predictability for coumarin-induced *in vivo* toxicity by using a toxicogenomics approach. Therefore, primary rat hepatocytes were cultured in sandwich configuration in medium containing a mixture of low concentrations of CYP450 inducers, phenobarbital, dexamethasone, and β-naphthoflavone. A toxicogenomics approach was employed enabling comparison of similar mechanistic endpoints at the molecular level between *in vitro* and *in vivo*, namely compound-induced changes in multiple genes and signaling pathways. Toxicant-induced cytotoxic effects and gene expression profiles observed in hepatocytes cultured in modified medium and hepatocytes cultured in standard medium (without inducers) were compared to results from a rat *in vivo* study. Coumarin was used as a model compound because its toxicity depends on bioactivation by CYP450 enzymes. Metabolism of coumarin towards active metabolites, coumarin-induced cytotoxicity, and gene expression modulation were more pronounced in hepatocytes cultured in modified medium compared to hepatocytes cultured in standard medium. Additionally, more genes and biological pathways were similarly affected by coumarin in hepatocytes cultured in modified medium and *in vivo*. In conclusion, these experiments showed that for coumarin-induced toxicity sandwich-cultured hepatocytes maintained in modified medium better represent the situation *in vivo* compared to hepatocytes cultured in standard medium.

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Introduction

To assess possible hepatotoxicity, conventional studies rely on the use of animal model systems to examine tissue toxin levels, changes in serum levels of hepatic enzymes, and histopathological changes (Nuwaysir et al., 1999; Waring and Ulrich, 2000). Simple, well established *in vitro* assays, such as primary hepatocytes, precision cut liver slices, and hepatic cell lines, are increasingly in demand for identifying potential hepatotoxicity in early stages of investigative toxicology and for decreasing attrition rates of drugs during lead optimization (Dambach et al., 2005). However, extrapolation of *in vitro* results to the *in vivo* situation remains a scientific challenge (Guillouzo, 1998).

Toxicogenomics, the application of the genomics technologies in toxicology, would be particularly useful in the extrapolation from *in vitro* experiments to the *in vivo* situation. Extrapolations can be made at the molecular level, comparing similar mechanistic endpoints, namely compound-induced changes in multiple genes and signaling pathways (Hamadeh et al., 2002; Stierum et al., 2005). Several toxicogenomics-based studies have already been performed comparing rat hepatic *in vitro* models with the situation *in vivo* (Waring et al., 2001; Boess et al., 2003; Jessen et al., 2003). These studies concluded that to date no toxicogenomics-based *in vitro* system allowed for prediction of hepatotoxic responses *in vivo*. The main limitation of hepatic *in vitro* assays used in these toxicogenomics-based studies is the loss of liver-specific functions, in particular cytochrome P450 (CYP450) monooxygenase activities (Balls et al., 2002; Boess et al., 2003). Extrapolation from these *in vitro* models to the *in vivo* situation is therefore hampered when examining compounds for which toxicity depends on bioactivation by the CYP450 enzyme system.

In the aim to develop an *in vitro* toxicogenomics-based system, the relevance of a hepatocyte sandwich culture with improved metabolic competence towards prediction of *in*

vivo toxicity was assessed. Sandwich-cultured hepatocytes were used because compared to other hepatocyte-based *in vitro* models, hepatocyte longevity is increased and a polarized cell and membrane architecture resembling *in vivo* is maintained for several weeks (Dunn et al., 1989; LeCluyse et al., 1996). Furthermore, rat hepatocytes maintained in sandwich configuration display a more optimal CYP450 inducibility (LeCluyse et al., 1999; Richert et al., 2002). In a separate study performed by this laboratory, it was shown that in sandwich-cultured hepatocytes maintained in modified medium, enriched with low concentrations of the known CYP450 inducers phenobarbital (PB), dexamethasone (DEX), and β-naphthoflavone (β-NF), the metabolic competence, as reflected by retention of CYP450 enzyme activities and gene expression levels of several phase I and phase II enzymes, was enhanced (Kienhuis et al., A sandwich-cultured primary rat hepatocyte system with increased metabolic competence evaluated by gene expression profiling, *submitted*). In the present study, the effect of coumarin (*cis*-*o*-coumaric acid lactone), a compound for which toxicity depends on metabolic activation by the CYP450 enzyme system (Born et al., 2002; Lake et al., 2002), on toxicity and gene expression profiles was studied in sandwich-cultured hepatocytes maintained in standard medium, the standard model (without inducers), and in sandwich-cultured hepatocytes maintained in modified medium, the modified model (containing enzyme inducers). Coumarin-induced cytotoxicity data and gene expression profiles generated in both *in vitro* models were compared to toxicity and gene expression profiles in rat liver in the *in vivo* study in which rats were exposed to coumarin, since *in vivo* verification of *in vitro* toxicogenomics data has been proposed to be a necessity to judge the value of the *in vitro* model for *in vivo* toxicity prediction (Jaeschke, 2003). The expected differences of bioactivation of coumarin in the standard model versus the modified model were verified by measurement of coumarin and one of its metabolites in medium.

Methods

Chemicals. Collagenase type B, Roche reagent kits, primer $p(DT)_{15}$, and Easyhyb were obtained from Roche (Mannheim, Germany). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), gentamycin, phosphate buffered saline (PBS), Trizol, yeast tRNA, human Cot-1 DNA were obtained from Invitrogen (Breda, The Netherlands). Insulin, glucagons, hydrocortisone, PB, DEX, β-NF, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and amino-allyl dUTP were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). The RNeasy mini kit, the RNAse-free DNAse kit, and the QIAquick PCR purification kit were obtained from Qiagen (Westburg B.V., Leusden, The Netherlands). Cy3 and Cy5 monofunctional reactive dyes, and Poly(dA)·Poly(dT) were purchased from Amersham Biosciences (Roosendaal, The Netherlands).
Coumarin, CAS-no 91-64-5; purity by HPLC minimum 99% according to the manufacturer, was obtained from Sigma-Aldrich. All other chemicals were of analytical grade.

Animals. Male Wistar rats (Crl: (WI) WU BR), 9-12 weeks of age, 180-250 g, were obtained from Charles River GmbH, Sulzfeld, Germany. During the acclimatization period and until sacrifice, animals were housed individually in macrolon cages with wire tops and sawdust bedding at 22^{o}C and 50-60% humidity. The light cycle was 12-h light / 12-h dark. Feed and tap water were available *ad libitum*.

Animal treatment *in vivo*. Wistar rats, housed under conditions as described above, were injected intraperitoneally (i.p.) with 17.5 mg/kg BW (low dose), 75 mg/kg BW (mid dose), and 200 mg/kg BW (high dose) coumarin dissolved in corn oil. Doses were defined in a range finding study. As a solvent control, only corn oil was injected (vehicle-treated control). In each dose group and vehicle-treated group, n=5 rats were included. Injection volume for each treatment was 10 mL/kg BW. Body weight was recorded on day 0 and at 24 hours, just

before sacrifice. Rats were anesthetized by inhalation of CO_2/O_2. Twenty-four hours after i.p. injection, animals were sacrificed by bleeding through the aorta abdominalis, the blood was collected in heparin tube, from which plasma was isolated for clinical chemistry. Thereafter, livers were immediately dissected, frozen in liquid nitrogen, and stored at -80^{o}C until further processing. A section of the liver was kept aside in formaline for pathological examination.

Clinical Chemistry. Serum alanine aminotransferase (ALAT) activity, serum aspartate aminotransferase (ASAT) activity, lactate dehydrogenase (LDH) leakage, serum alkaline phosphatase (ALP) activity, glucose, cholesterol, γ-glutamyl transferase (GGT) activity, and phospholipid levels were analyzed on a Hitachi 911 centrifugal analyzer using Roche reagent kits. Differences between plasma levels in treated and non-treated animals were defined as statistically significant at a p-value below 0.01, determined by one-way ANOVA followed by Dunnett's test.

Culture of rat hepatocytes. Male Wistar rats similar to those used in the *in vivo* study and housed under identical conditions were used for hepatocyte isolation. Hepatocytes were isolated from livers from three individual rats according to a two-step collagenase perfusion technique as described by Seglen (Seglen, 1976; Paine et al., 1979) with minor modifications (Paine et al., 1979). Hepatocyte preparations with viability greater than 85% as determined by Trypan Blue exclusion were used and cultured on collagen gel precoated 6-well plates at a density of 1.25 x 10^6 cells per well. Sandwich cultures were essentially prepared according to the method of Beken et al. . Hepatocytes were allowed to attach for 4 hours in DMEM supplemented with 10% FCS, insulin (0.5 U/ml), glucagon (7 ng/ml), gentamycin (50 μg/ml). After attachment, dead cells were removed by washing and the upper collagen layer was applied. Cells were kept in standard medium consisting of DMEM containing 25 mM HEPES and 4.5 g/l D-glucose supplemented with insulin (0.5 U/ml), glucagon (7 ng/ml), hydrocortisone (7.5 μg/ml), and gentamycin (50 μg/ml). In the modified model, standard

culture medium was modified by supplementation of an inducer mix that consisted of 1 mM PB, 10 µM DEX, and 5 µM β-NF (Kienhuis et al., A sandwich-cultured primary rat hepatocyte system with increased metabolic competence evaluated by gene expression profiling, *submitted*). PB was added as a concentrated stock solution in PBS. DEX and β-NF were added as concentrated stock solutions in dimethylsulphoxide (DMSO). The final concentration of DMSO was equalized in all culture media and did not exceed 0.2% (v/v). Standard and modified media were applied four hours after seeding the cells. Cultures were incubated at 37 ^{o}C in a humidified incubator, O_2 95% and CO_2 5%. Medium was changed on a daily basis during a period of 72 hours.

Hepatocyte treatment and cytotoxicity analysis. Hepatocytes cultured in either standard or modified medium for 72 hours were exposed to coumarin in a concentration range of 0 to 1000 µM dissolved in DMSO for 24 hours. The final concentration of DMSO was 0.5% (v/v). Cytotoxicity was determined employing the MTT reduction method (Mosmann, 1983). LDH leakage was determined spectrophotometrically in pooled medium obtained from three wells per rat per dose group, on a Hitachi 911 centrifugal analyzer using Roche reagent kits. The final coumarin concentrations for the gene expression study selected were 0 µM (control), 70 µM (low dose), 200 µM (mid dose), and 600 µM (high dose). Doses corresponded to 0%, 10%, 20%, and 50% cytotox as determined by the MTT reduction assay, respectively (see results).

Gas chromatography to study metabolism of coumarin. Coumarin and the *o*-hydroxyphenylacetic acid (*o*-HPAA) metabolite were measured in standard and modified culture medium of hepatocytes exposed to coumarin for 24 hours using gas chromatography with flame ionization detection (GC-FID) as described (Born et al., 2000a; Born et al., 2003). Samples (0.1 ml) were diluted with water to a final volume of 1 ml. To increase the extraction efficiency of *o*-HPAA, 0.5 g sodium chloride was added to each sample, following extraction

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with ethyl acetate. The extraction efficiencies of coumarin and *o*-HPAA were 30% and 65%, respectively. CM and *o*-HPAA were separated and quantitated using a Fisons HRGC 8650 gaschromatograph and a Varian VG-5ms column, 50 m x 0.25 mm. Quantification was accomplished by calculation of peak-area ratios relative to the *n*-tridecane (*n*-C13) internal standard, and use of the external standard curves. The same extraction procedure was applied to the external standards.

Total RNA isolation. RNA was extracted using Trizol according to the manufacturer's protocol. Trizol was added to frozen liver samples obtained from the *in vivo* study which subsequently were pulverized with mortar and pestle under liquid nitrogen before extraction. To obtain RNA from sandwich cultures, Trizol was added on the upper collagen layer, and cells were collected. RNA was purified using the RNeasy mini kit including an additional DNAse digestion step. RNA concentration was calculated from the absorbance at 260 nm as measured spectrophotometrically. RNA quality was assessed by agarose gel electrophoresis.

Microarray design. A local reference design was used for microarray hybridization (Table 1). For the *in vivo* samples, tester RNA labeled with Cy5 from individual rat livers was combined with Cy3-labeled reference RNA consisting of a pool of RNA extracted from livers obtained from five vehicle-treated, control rats. For the *in vitro* studies, Cy5 labeled tester RNA from one experiment was combined with Cy3 labeled reference RNA from the same experiment; hepatocytes in one experiment were obtained from one rat.

Microarray labeling. RNA samples were indirectly labeled according to the amino-allyl labeling procedure for microarrays from The Institute for Genomic Research (TIGR) (http://www.tigr.org/tdb/microarray/protocolsTIGR.shtml). For each labeling reaction, 25 µg of total RNA was used as starting amount for reverse transcription of mRNA. Briefly, for reverse transcription, mRNA was selected by oligo dT priming with primer $p(DT)_{15}$. The

reverse transcription reaction in which amino-allyl dUTP is incorporated, was conducted for three hours at 42 ^{o}C. Non-transcribed RNA was degraded by alkaline hydrolysis in a final concentration of 0.25 M NaOH for 30 minutes 37 ^{o}C. Thereafter, the mixture was neutralized with an equimolar amount of acetic acid. The cDNA was purified using the Qiagen QIAquick PCR purification kit. Columns were washed with 10 mM sodium-borate in 80% ethanol (pH 8.5). Column-bound cDNA was eluted two times in 30 µl MilliQ H_2O. Samples were labeled with either Cy3 (reference) or Cy5 (tester) monofunctional reactive dyes and afterwards cleaned from unincorporated Cy dyes using AutoseqG-50 sephadex chromatography columns.

Microarray hybridization. Samples were hybridized on rat oligonucleotide microarrays containing approximately 5800 different oligonucleotide fragments of 70 nucleotides in length (Qiagen Operon, Westburg B.V., Leusden, The Netherlands), spotted in duplicate at the Frank Holstege group (Utrecht genomics laboratory, Utrecht, The Netherlands) as described (van de Peppel et al., 2003; Heijne et al., 2005). Briefly, Cy3 and Cy5 labeled samples were combined according to the experimental design. To avoid non-specific hybridization, yeast tRNA, Poly(dA)·Poly(dT) and human Cot-1 DNA were added. Samples were dissolved in 110 µl Easyhyb hybridization buffer. After cDNA denaturation at 100 ^{o}C, human Cot-1 DNA was allowed to anneal for half an hour at 42 ^{o}C. The hybridization mixture was pipetted directly in the center of the hybridization chamber (Corning Life Sciences B.V., Schiphol-Rijk, The Netherlands). Slides were prehybridized according to the TIGR protocol and placed carefully on top. To keep the chamber moisturized during hybridization, a pre-cut paper drenched in MilliQ H_2O was put on top. Samples were hybridized overnight at 42 ^{o}C in a water bath. Slides were washed with sodium chloride sodium citrate (SSC) buffers decreasing in stringency. Microarrays were scanned using a Packard Scanarray confocal laser scanner (PerkinElmer, Boston, MA). Resulting TIFF images were loaded into Imagene 5.0 (Biodiscovery Inc. El Segundo, CA) and saved to further process and analyze the data.

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Microarray quality criteria. Criteria for microarrays to be further analyzed consider the homogeneity of the spot signal intensities, the effect of bleaching of the fluorescence, the number of manually flagged (excluded) spots, the spatial distribution of the signals over the slide surface, the balance between Cy3 and Cy5 signal intensity, the number of saturated spots and the quality of the slide with respect to other slides of the experiment, as described by Heijne et al. .

Microarray data analysis. Flagged spots and controls were excluded from further analyses. Only spots on qualified microarrays with intensities higher than 1.5 times the intensity of their local background were included in data analysis. Ratios of the background-corrected intensities of tester over reference were calculated for each slide. To account for technical variations introduced during labeling or hybridization, data were normalized using the lowess algorithm (Yang et al., 2002). Normalized ratios were log-transformed with base two in SAS Enterprise guide V2 (SAS Institute Inc., Cary). The resulting data set was loaded into Excel (Microsoft Corporation, Redmond, WA). Replicate genes per array were averaged. For a gene to be included in the analysis for both *in vivo* and *in vitro* studies, a maximum of 30% missing values per gene were accepted. Two sample *t*-tests (with random variance model) were performed using BRB-Array Tools Version 3.3.1 developed by Dr. R. Simon and A. Peng Lam (Simon and Peng, 2002). To reduce false positives discovery, the nominal significance level of every univariate test was set at 0.001. To study whether similarities between biological processes could be observed between the *in vitro* models and *in vivo*, pathway analysis was performed using genes significantly expressed as determined by BRB ArrayTools, Genelists were uploaded on the website of the Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://apps1.niaid.nih.gov/david/). Pathway analyses were performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.ad.jp/kegg). A pathway was considered “triggered” when at least two

genes within the pathway were significantly modulated by coumarin as assessed by means of the Fisher's exact test. Therefore, for each pathway and per system (*in vitro* and *in vivo*), the total number of genes that gave a signal on the microarray was divided into four groups: the number of genes not significantly modulated by coumarin and representing the pathway, not significantly modulated by coumarin and not representing the pathway, significantly modulated by coumarin and representing the pathway, and finally, significantly modulated by coumarin and not representing the pathway. Pathways were considered significant at a p-value below 0.05. Furthermore, principal component analysis (PCA) was performed using Matlab software Version 6.5 (The MathWorks Inc., Natick).

Results

In vivo *toxicity.* Hepatotoxicity *in vivo* was determined in rats treated with coumarin up to 200 mg/kg BW. Clinical chemistry parameters showed that coumarin significantly increased the plasma levels of cholesterol at a dose level of 75 mg/kg BW, and AST, ALT, and GGT at a dose of 200 mg/kg BW. The phospholipids level measured in plasma of rats exposed to 200 mg/kg BW coumarin was significantly decreased as presented in Table 2 (data represent mean ± SD). Histopathological findings in rat liver show that at necropsy, the liver of one animal treated with 75 mg/kg BW of coumarin was focally discolored, probably by focal congestion. Upon microscopy, only animals treated with 200 mg/kg showed slight to severe single cell necrosis and minimally, centrilobular and mononuclear cell infiltrate.

***In vitro* toxicity.** Cytotoxicity of coumarin was determined in the sandwich-cultured hepatocyte model containing either standard medium or modified medium (Fig. 1). No cytotoxicity of coumarin (up to 1 mM) was detected in the standard system. However, in the modified system coumarin appeared to be cytotoxic in a dose-response manner. Based on these data, for the gene expression study, hepatocytes were exposed to coumarin at 0 µM (control), 70 µM (low dose), 200 µM (mid dose), and 600 µM (high dose) for 24 hours. In the modified system, the low, mid, and high dose resulted in 100-90%, 90-80%, and <50% viability, respectively. In the standard system, the viability was 100% at all concentrations of coumarin.

Coumarin metabolism. To verify that the improved metabolic competence of the modified model indeed affects the metabolism of coumarin and thus is responsible for differences in cytotoxicity of coumarin in standard culture medium and modified culture medium, both coumarin and the major coumarin metabolite reported to be detected in rat urine (Lake, 1999; Born et al., 2000a; Born et al., 2000b) were measured in both standard and

modified culture medium 24 hours after exposure. A dose dependent increase in the formation of *o*-HPAA was exclusively measured in the modified culture medium, but no *o*-HPAA was present in the standard culture medium. Furthermore, for all dose groups, coumarin concentration was significantly lower in the modified medium compared to the standard medium (the reduction varied from 15% for the high dose group to 59% for the low dose group; data not presented).

Gene expression profiling.

Analysis of significantly modulated genes. In total, the criteria settings for microarray analysis used in this study resulted in datasets containing gene expression ratios of 1621 genes *in vivo*, 2536 in the standard system, and 2368 genes in the modified system. The large volume of data in the dataset was reduced by performing robust statistical analysis that separated genes which expression was actually changed by coumarin from genes that remained unchanged. Resulting datasets contained, regardless of dose-dependency, 321, 13, and 92 genes which significantly changed by coumarin *in vivo*, in the standard system, and the modified system, respectively. Overlap of the changed genes between both *in vitro* models and *in vivo* is presented by the Venn diagram in Fig. 2. Twenty-three genes were altered in both the modified model and *in vivo*, and only three genes in both the standard system and *in vivo*. One gene was altered in all systems, *in vitro* and *in vivo*. These 27 genes form the subset of genes significantly changed by coumarin which are listed in Table 3. In both *in vivo* and the *in vitro* models, most genes showed a dose-dependent down regulation (for 52 to 85% of the genes the Pearson correlation analysis coefficient is <-0.8; data not shown). When comparing the different systems with each other, the direction of modulation was always comparable between the standard and the modified *in vitro* models, and in most of the cases also between the *in vivo* model and one of the *in vitro* models.

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Pathway analysis. Pathways triggered by coumarin in at least two systems and significant in at least one are presented in Table 4. In the standard model, the modified model, and *in vivo*, one, eight, and six pathways were significantly triggered by coumarin, respectively. Four pathways, i.e. methionine metabolism, fatty acid metabolism, gamma-hexachlorocyclohexane degradation, and complement and coagulation cascades, were significant in both the modified system and *in vivo*. Only one pathway was significantly triggered in the standard system, but not *in vivo*.

Principal component analysis of whole data set. The complete datasets of the *in vivo* and *in vitro* studies were used for PCA (Fig. 3). The two major components within the total variation between *in vivo* and both *in vitro* models, principal component (PC) #1 and PC #2, explain 40% of the variance in the data set. The results show that genomics responses induced by the low and mid dose in the standard system could not be distinguished from controls, whereas a dose-response effect of coumarin *in vivo* and in the modified system was evident along the PC#2 axis, explaining14% of the variance in the whole data set. Along the PC#1 axis, 26% of the variance in the data was explained by genes reacting differently *in vivo* and *in vitro*.

Discussion

To investigate the relevance of hepatocyte sandwich cultures towards mimicking aspects of *in vivo* toxicity, cytotoxicity measures and gene expression profiles were compared with data from an *in vivo* rat study. Coumarin, a compound for which toxicity depends on metabolism by the CYP450 enzyme system, was used as a model compound. Specifically, CYP450 enzymes of the 1A and 2E subfamily convert coumarin to the toxic metabolite coumarin 3,4-epoxide (CE) (Born et al., 2002; Lake et al., 2002). CE rearranges spontaneously to the more stable *o*-hydroxyphenylacetaldehyde (*o*-HPA). Both CE and *o*-HPA are assumed to contribute to the hepatotoxicity of coumarin in rats as they conjugate with critical cellular macromolecules (Lake et al., 1989; Fentem and Fry, 1993; Lake et al., 1994; Born et al., 1997; Born et al., 2000b; Born et al., 2003).

Prior to engaging into gene expression studies, it was hypothesized that an increased metabolic competence of sandwich cultures would improve metabolic conversion of coumarin to toxic metabolites, thereby increasing the resemblance with aspects of *in vivo* coumarin-induced hepatotoxicity. Therefore, sandwich-cultured hepatocytes were either maintained in a standard medium or in a modified medium that was enriched with a mixture of low concentrations of known CYP450 inducers in order to enhance metabolic capacity (Kienhuis et al., A sandwich-cultured primary rat hepatocyte system with increased metabolic competence evaluated by gene expression profiling, *submitted*). Findings in this study showed that both traditional measures of toxicity and coumarin-induced gene expression in the modified model were more pronounced and closer to *in vivo* compared to the standard model.

In vivo, coumarin toxicity was indicated by increased plasma ALT, AST, and GGT levels, primarily at the highest dose level of 200 mg/kg BW. Moreover, hepatotoxicity did manifest itself as severe single cell necrosis and minimal centrilobular necrosis, as observed

by histopathological observations in livers of rats treated with 200 mg/kg coumarin. These findings agree with other studies which report that administration of coumarin in doses ranging from 125 to 500 mg/kg results in severe centrilobular necrosis after 24 hours (Lake et al., 1989; Lake et al., 1994; Lake, 1999).

In vitro, coumarin appeared to be cytotoxic in hepatocytes cultured in modified medium, whereas no cytotoxicity was measured in the standard model. In our laboratory, it has been shown that gene expression of *CYP1A2* and *CYP2E1* in the modified model are closer to *in vivo* compared to the standard model in which gene expression of these genes is highly downregulated. Furthermore, upon addition of the inducer mixture, enzyme activities of the 1A subfamily were increased to levels comparable to *in vivo* (Kienhuis et al., A sandwich-cultured primary rat hepatocyte system with increased metabolic competence evaluated by gene expression profiling, *submitted*). As coumarin is converted by CYP1A and CYP2E to *o*-HPA (Born et al., 2002; Lake et al., 2002), this metabolite possibly causes the cytotoxicity in the modified model. It was shown earlier that the compounds in the inducer mixture, PB, DEX, and β-NF increase the formation of *o*-HPA and other coumarin metabolites, enhancing coumarin toxicity (Peters et al., 1991; Fentem and Fry, 1992). In the present study, these findings were confirmed by measurement of the coumarin metabolite *o*-HPAA, which is formed after oxidation of *o*-HPA. After 24 hours of coumarin exposure, a dose dependant formation of *o*-HPAA was exclusively observed in hepatocytes cultured in the modified model, whereas no *o*-HPAA was formed in the standard model. Furthermore, coumarin clearance was significantly higher in the modified culture medium compared to the standard culture medium in all dose groups.

Analysis of gene expression revealed that both *in vivo* and *in vitro*, genes are affected by coumarin at dose levels where no toxicity occurs as determined by traditional toxicology measures. This suggests that gene expression changes may very well be more sensitive

indicators of potential adverse effects, as was indicated before by Heinloth et al. (Heinloth et al., 2004). Considerably more genes were significantly altered and similarly affected by coumarin in both the modified system and *in vivo* compared to the standard system. In this study, not all affected genes are discussed in detail. It is of more relevance to investigate analogies in the biological pathways these genes trigger. The four pathways that were statistically significantly triggered both *in vivo* and in the modified model and the significant differentially expressed genes within these pathways will be discussed.

Betaine-homocysteine methyltransferase (*Bhmt*) and CTL target antigen (*Cth*), a putative cystathione gamma lyase enzyme, both downregulated *in vivo* and in the modified system, appear in the methionine metabolism pathway. It has been shown that downregulation of *Bhmt* and *Cth* impairs conversion of homocysteine to methionine and cysteine, respectively, resulting in increased homocysteine levels in the cell (Torres et al., 1999; Forestier et al., 2003). Homocysteine accumulation is associated with impaired liver function, necrosis, liver cirrhosis, and fibrogenesis (Torres et al., 1999; Finkelstein, 2003).

Expression of *CYP1A2*, a gene which represents both the gamma-hexachlorocyclohexane degradation pathway and the fatty acid metabolism pathway, is significantly downregulated *in vivo* and in the modified model. This CYP450 belongs to the CYP1A subfamily that converts coumarin to toxic metabolites. Therefore, downregulation of gene expression of this enzyme could be interpreted as a classical negative feedback loop, turning out in the present case to prevent additional liver injury.

Fibrinogen gamma polypeptide (*fgg*) is significantly upregulated *in vivo* and significantly downregulated in the modified model. This gene is part of the complement and coagulation cascade, to which coumarin is associated as coumarin derivatives, such as warfarin, are well known for their anticoagulant properties (Egan et al., 1990). One possible explanation of the significant, but different response of particular genes to coumarin *in vivo*

and *in vitro* can be illustrated by *fgg*. *Fgg* is mainly expressed in hepatocytes. Upregulation of *fgg*, as occurred *in vivo*, would be expected in terms of toxicity as fibrinogen levels are increased following liver injury (Redman and Xia, 2001). Fibrinogen synthesis has shown to be stimulated in hepatocytes by factors excreted by extrahepatic tissues or nonparenchymal cells (Otto et al., 1987). *In vitro*, these factors are absent. Therefore, stimulation of fibrinogen synthesis in hepatocyte cultures *in vitro* may only occur when these factors are added to the culture medium.

Additional to the statistical analysis of microarray data which resulted in a list of significantly modulated genes, PCA was performed. PCA allows inclusion of all genes into analysis, preserving their interrelationships. Results showed that even though differences between *in vitro* and *in vivo* remain evident, the dose-response effect with respect to gene expression of coumarin in the modified system is more similar to *in vivo* compared to the standard system.

Dose levels *in vivo* were compared with the coumarin concentrations applied *in vitro*. Using pharmacokinetic data from literature, a distribution coefficient of coumarin of 3.33 L/kg was estimated (Ritschel and Hussain, 1988; Lake, 1999; Born et al., 2003). Assuming that *in vivo* bioavailability of coumarin is 100% after i.p. injection, one can estimate a maximum *in vivo* plasma concentration of 36 µM, 154 µM, and 411 µM for the low, mid, and high *in vivo* doses, respectively. These concentrations are in the same range as the *in vitro* concentrations of 70 µM, 200 µM, and 600 µM. Although plasma levels of coumarin were not determined *in vivo*, the range of estimated maximum plasma levels *in vivo*, and therefore potential target organ concentrations, were comparable to the dose level range employed in the culture media *in vitro*.

It is important to note that in addition to coumarin, the inducer mixture may also influence gene expression and that these effects interact with each other. In the design of the

present study, however, effect of the inducer mixture on gene expression was filtered out because matching controls were used, i.e. both test and control samples are from hepatocytes cultured in the modified system. Nonetheless, it remains possible that the inducer mixture affected the expression of some genes in the modified system in such way that no additional effect of coumarin could have been detected.

Furthermore, effects on gene expression in the highest dose group of the modified model may not be solely attributed to coumarin. The highest coumarin dose group in this model resulted in a 50% loss of cell viability as measured by the MTT reduction assay. In a sandwich configuration, dead cells remain between the two layers of collagen. As a consequence, necrosis caused by coumarin exposure in one cell can affect gene expression in viable neighbor cells (Fielden and Zacharewski, 2001). Excluding the high dose group of the modified model from analysis, still 80% of the genes in Table 2 are retained. Moreover, the methionine metabolism and gamma-hexachlorocyclohexane degradation pathways are still significantly triggered by coumarin.

In summary, our experiments have shown that the metabolism of coumarin towards active metabolites, coumarin-induced toxicity, gene expression profiles, and consequently, biological pathways in the modified system containing sandwich-cultured hepatocytes with enhanced metabolic capacity better represent the situation *in vivo* compared to conventional sandwich-cultured hepatocytes. This highlights the need for a metabolically competent, toxicogenomics-based, hepatocyte *in vitro* system.

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Footnotes

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Person to receive reprint requests

Rob H. Stierum, TNO Quality of Life, Business Unit Biosciences, Physiological Genomics, P.O. Box 360 (PP 8), 3700 AJ, Zeist, The Netherlands. Telephone: +31 30 694 4545. Telefax: +31 30 696 0264. E-mail: rob.stierum@tno.nl.

Legends for figures

Fig. 1. Cytotoxicity of coumarin in the standard model (A) and the modified model (B). MTT reduction is expressed as a percentage of the MTT determination of control hepatocyte cultures incubated with vehicle only. LDH retention is expressed as an inverted percentage of the LDH leakage in Triton-X treated hepatocyte cultures. Data are means ± SD (n=3).

Fig. 2. Circles in de Venn diagram represent the number of genes significantly changed *in vivo*, in the standard model (ST), and in the modified model (MOD). Overlaps contain the number of significantly modulated genes similar between systems.

Fig. 3. Principal component analysis of the complete dataset of expression profiles resulting from *in vitro* and *in vivo* hepatotoxicant-treatment of hepatocytes and rats. Two principal components were generated and plotted for each system and dose group. Dose groups are distinguished by circles and identified within (*in vivo*) or outside (ST, the standard model; MOD, the modified model) each circle.

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TABLE 1

Experimental design

<table>
<tr><th>in vivo</th><th colspan="3">in vitro</th></tr>
<tr><td rowspan="3"></td><td>Experiment 1
Rat 1</td><td>Experiment 2
Rat 2</td><td>Experiment 3
Rat 3</td></tr>
<tr><td colspan="3">Isolation of hepatocytes and preparation of collagen sandwich cultures for each rat</td></tr>
<tr><td colspan="3">Incubation into standard or modified medium</td></tr>
<tr><td>in vivo exposure to coumarin for 24h at 0, 17.5, 75, and 200 mg/kg BW; n=5 per dose group</td><td colspan="3">Exposure of cultures to coumarin for 24h at tester concentrations of 0 (reference control), 70, 200, and 600 µM</td></tr>
<tr><td>Cy5 labeling of RNA from each individual coumarin treated rat</td><td colspan="3">Cy5 labeling of RNA from coumarin treated cultures</td></tr>
<tr><td>Cy3 labeling of pool RNA from control animals</td><td colspan="3">Cy3 labeling of pool of RNA from respective control cultures</td></tr>
<tr><td colspan="4">Resulting datasets from in vivo and in vitro cultures combined</td></tr>
<tr><td colspan="4">Maximum missing values allowed:
2 out of 5 for the in vivo study
1 out of 3 for the in vitro studies</td></tr>
</table>

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TABLE 2

Clinical chemistry parameters from n=5 coumarin-treated rats per treatment group in the in vivo *study*

	Control	Low dose	Mid dose	High dose
Coumarin dose	0 (mg/kg BW)	17.5 (mg/kg BW)	75 (mg/kg BW)	200 (mg/kg BW)
ALP (U/l)	136 ± 13	151 ± 20	139 ± 31	163 ± 24
Cholesterol (mM)	1.71 ± 0.13	1.65 ± 0.21	2.26 ± 0.4**	1.38 ± 0.26
Glucose (mM)	10.41 ± 0.26	10.45 ± 0.35	10.72 ± 1.56	10.26 ± 0.89
ASAT (U/l)	68 ± 13	81 ± 21	59 ± 3	383 ± 244**
ALAT (U/l)	66 ± 21	61 ± 20	35 ± 3	264 ± 175**
GGT (U/l)	1.4 ± 0.4	1.6 ± 0.4	1.5 ± 0.3	3.3 ± 1.7**
LDH (U/l)	168 ± 92	342 ± 1.99	126 ± 26	421 ± 154
Phospholipids	1.93 ± 0.12	1.73 ± 0.18	1.94 ± 0.27	1.44 ± 0.24**

***p < 0.01 as determined by ANOVA followed by Dunnett's test.*

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TABLE 3

Genes significantly changed by coumarin in at least two systems; in vivo, *in the standard, or in the modified model*

			in vivo (mg/kg BW)			Standard *in vitro* model (µM)			Modified *in vitro* model (µM)		
			17.5	75	200	70	200	600	70	200	600
AccNumber	GeneName	Symbol	Low	Mid	High	Low	Mid	High	Low	Mid	High
NM_030850	betaine-homocysteine methyltransferase	Bhmt	**-1.00**	**-2.41**	**-1.63**	0.17	-0.58	-1.82	**-1.55**	**-1.53**	-1.08
M35266	cytosolic cysteine dioxygenase 1	Cdo1	0.14	**0.69**	-0.35	-0.25	-0.81	-1.36	**-0.66**	-2.05	**-2.38**
NM_017074	CTL target antigen	Cth	-0.09	-0.34	**-2.21**	0.05	-0.49	-1.18	**-0.62**	-1.07	**-1.18**
M13646	testosterone 6-beta-hydroxylase	CYP3A2	0.52	0.27	**-1.03**	xxx	xxx	xxx	**-0.58**	-0.42	-0.32
X69834	serine (or cysteine) proteinase inhibitor, clade A, member 3M	Serpina3m	-0.01	-0.23	**-0.93**	-0.02	-0.14	-0.40	**-0.75**	-0.72	-0.55
NM_130433	acetyl-Coenzyme A acyltransferase 2	Acaa2	0.08	**0.68**	0.37	0.01	-0.25	-0.75	**-0.79**	-1.60	-1.86
NM_017321	iron-responsive element-binding protein	Ratireb	0.25	**0.88**	0.23	0.11	0.14	-0.07	-0.47	**-1.07**	-1.04
NM_080892	selenium binding protein 2	Selenbp2	-0.39	-0.39	**-1.33**	-0.09	-0.02	-0.70	-0.87	**-2.18**	-2.71
NM_012541	cytochrome P450, 1a2	Cyp1a2	-0.15	-0.28	**-1.28**	-0.14	-0.36	-0.23	-0.92	**-2.08**	**-3.01**
NM_017306	dodecenoyl-coenzyme A delta isomerase	Dci	0.62	**1.91**	**1.49**	0.05	-0.01	-0.17	-0.18	**-0.59**	-0.79
NM_012826	alpha-2-glycoprotein 1, zinc	Azgp1	0.12	0.16	**-0.87**	-0.20	**-0.50**	**-0.75**	-0.48	**-1.06**	-1.60
D17310	3-alpha-hydroxysteroid dehydrogenase		0.08	**0.50**	-0.18	0.15	0.17	-0.33	-0.75	**-2.13**	-2.70
NM_031736	solute carrier family 27 (fatty acid transporter), member 32	Slc27a2	0.41	**1.42**	0.72	-0.02	-0.34	-0.41	-0.43	**-0.99**	-1.15

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TABLE 3

Continued

			Liver *in vivo* (mg/kg BW)			Standard *in vitro* model (µM)			Modified *in vitro* model (µM)		
			17.5	75	200	70	200	600	70	200	600
AccNumber	GeneName	Symbol	Low	Mid	High	Low	Mid	High	Low	Mid	High
NM_031760	ATP-binding cassette, sub-family B (MDR/TAP), member 11	Abcb11	0.00	0.11	**-0.93**	-0.15	-0.27	-0.66	-0.70	**-1.53**	**-1.90**
NM_030826	glutathione peroxidase 1	Gpx1	0.11	-0.44	**-1.29**	-0.09	-0.21	-0.80	-0.72	**-1.25**	**-1.43**
NM_138884	aldo-keto reductase family 1, member D1	Akr1d1	0.48	**1.48**	0.92	-0.06	-0.35	-0.93	-0.47	**-1.21**	-1.18
NM_012699	dnaJ homolog, subfamily b, member 9	Dnajb9	0.20	0.16	**1.02**	-0.10	-0.11	0.72	0.27	**1.35**	2.04
AB009463	low density lipoprotein receptor-related protein 3	Lrp3	-0.08	-0.27	**-0.45**	-0.05	-0.09	-0.45	-0.50	**-1.00**	xxx
NM_022513	dopa/tyrosine sulfotransferase		-0.13	**-0.47**	-0.24	0.04	-0.19	-0.67	-0.74	**-1.33**	-1.04
D50559	sterol-C4-methyl oxidase-like	Sc4mol	0.01	0.25	**-1.20**	-0.02	0.02	-0.68	-0.32	-0.84	**-1.30**
X60822	methionine adenosyltransferase I, alpha	Mat1a	-0.51	**0.76**	0.25	0.07	-0.53	-0.80	-0.68	-2.20	**-2.52**
NM_012559	Fibrinogen, gamma polypeptide	Fgg	0.35	1.08	**1.19**	0.10	-0.35	-0.92	-0.78	-0.89	**-0.58**
NM_016987	ATP citrate lyase	Acly	0.01	-1.11	**-1.93**	0.05	-0.28	-0.61	-0.10	-0.69	**-1.00**
NM_017134	arginase 1	Arg1	-0.18	-0.13	**0.76**	-0.40	-0.16	-0.23	-0.67	-1.33	**-1.63**
NM_017332	fatty acid synthase	Fasn	-0.62	**-2.06**	**-2.56**	0.05	-0.56	**-1.05**	-0.02	0.09	-0.16
K01934	thyroid hormone responsive protein	Thrsp	-1.02	**-3.08**	**-3.47**	-0.08	-1.07	**-2.05**	-1.19	-1.07	-0.64
X56228	thiosulfate sulfurtransferase	Tst	0.00	-0.49	**-1.11**	-0.08	-0.17	**-0.51**	-0.10	-0.73	-1.46

Values are log transformed ratios corrected for the control; significant values ($p < 0.001$) are marked in bold.

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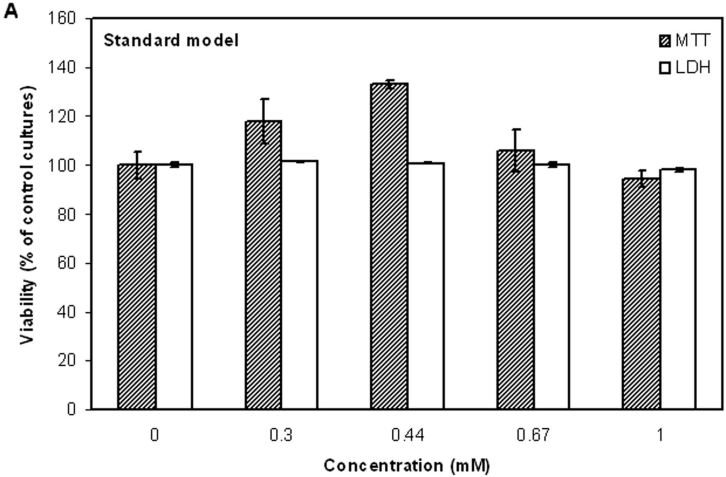
TABLE 4

Pathways triggered by coumarin in vivo*, in the standard model, or in the modified model*

	in vivo		Standard model		Modified model	
Pathway	significant/ signal	p-value	significant/ signal	p-value	significant/ signal	p-value
Fatty acid metabolism	12 / 34	0.024*	0 / 35	1.000	4 / 36	0.049*
Tryptophan metabolism	11 / 28	0.013*	0 / 26	1.000	3 / 27	0.085
Complement and coagulation cascades	10 / 28	0.036*	0 / 29	1.000	4 / 27	0.019*
Citrate cycle (TCA cycle)	6 / 11	0.011*	0 / 11	1.000	2 / 11	0.065
Gamma-Hexachlorocyclohexane degradation	8 / 15	0.004*	0 / 14	1.000	3 / 14	0.015*
Urea cycle and metabolism of amino groups	2 / 8	0.492	0 / 9	1.000	2 / 9	0.045*
Nitrogen metabolism	3 / 8	0.199	0 / 7	1.000	2 / 7	0.028*
Selenoamino acid metabolism	2 / 3	0.102	0 / 7	1.000	2 / 7	0.028*
Cysteine metabolism	3 / 6	0.096	0 / 6	1.000	2 / 5	0.014*
Methionine metabolism	3 / 4	0.026*	0 / 6	1.000	3 / 6	0.001*
Glutathione metabolism	4 / 13	0.246	2 / 16	0.003*	1 / 15	0.449

**p < 0.05 calculated by the Fisher's exact test; the significant / signal column represents the number of genes in the pathway significantly altered by coumarin versus the number of genes in that pathway present in the complete dataset.*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Standard model
MTT
LDH
Viability (% of control cultures)
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140
120
100
80
60
40
20
0
0
0.3
0.44
0.67
1
Concentration (mM)

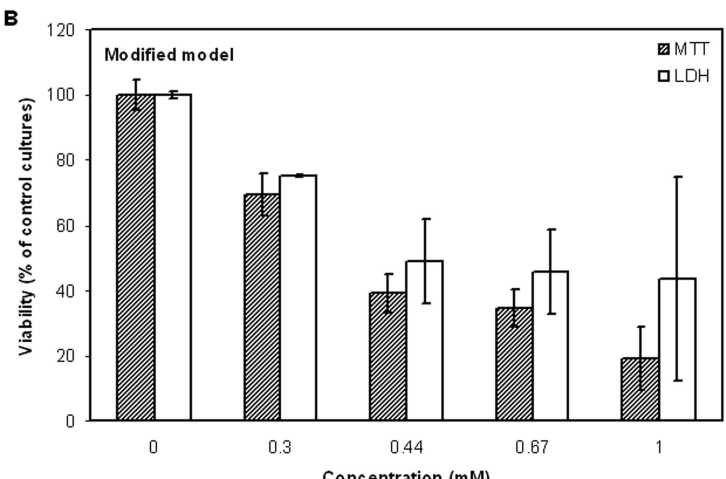
B
Modified model
MTT
LDH
Viability (% of control cultures)
120
100
80
60
40
20
0
0
0.3
0.44
0.67
1
Concentration (mM)

Fig. 1

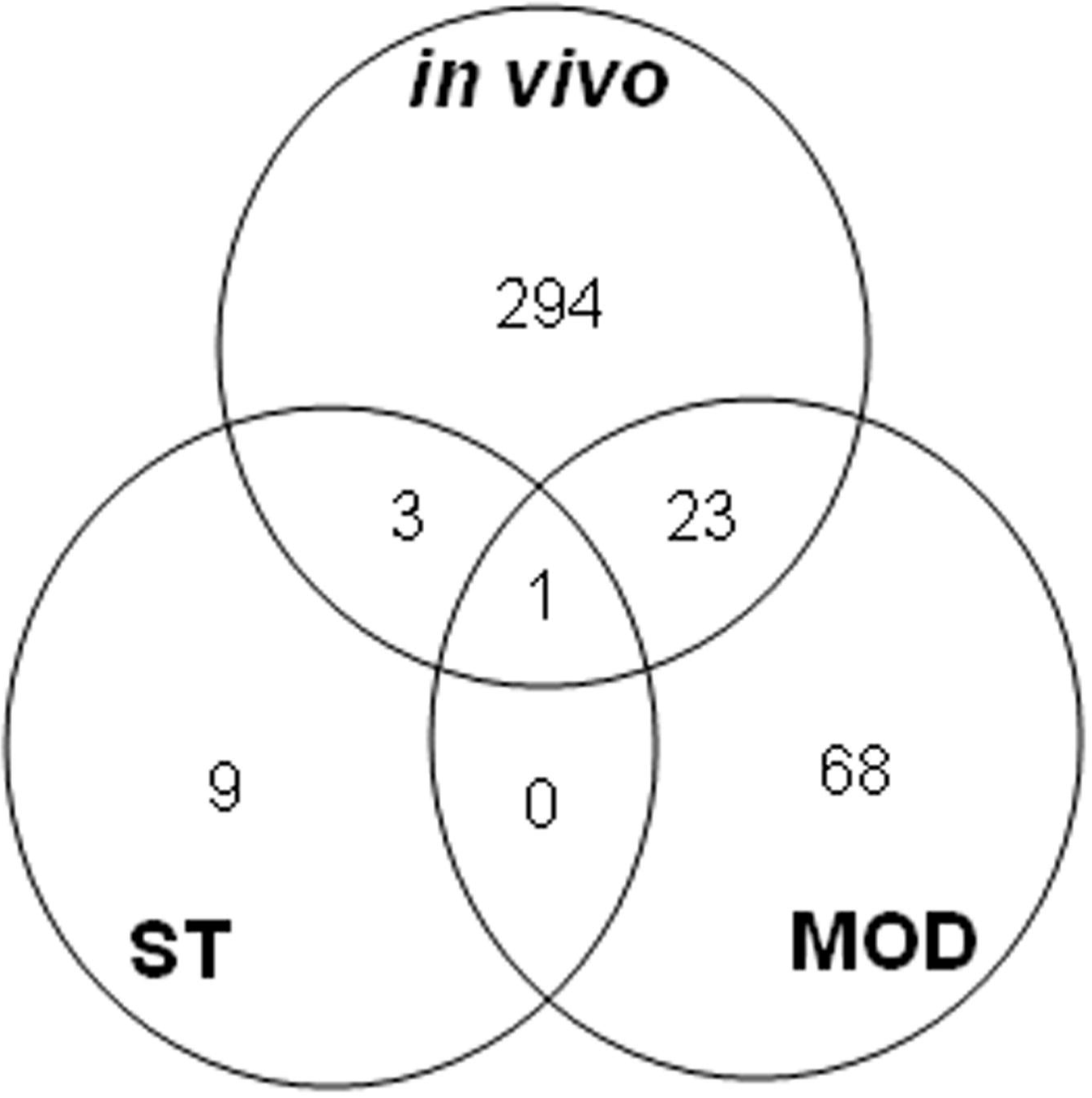

Fig. 2

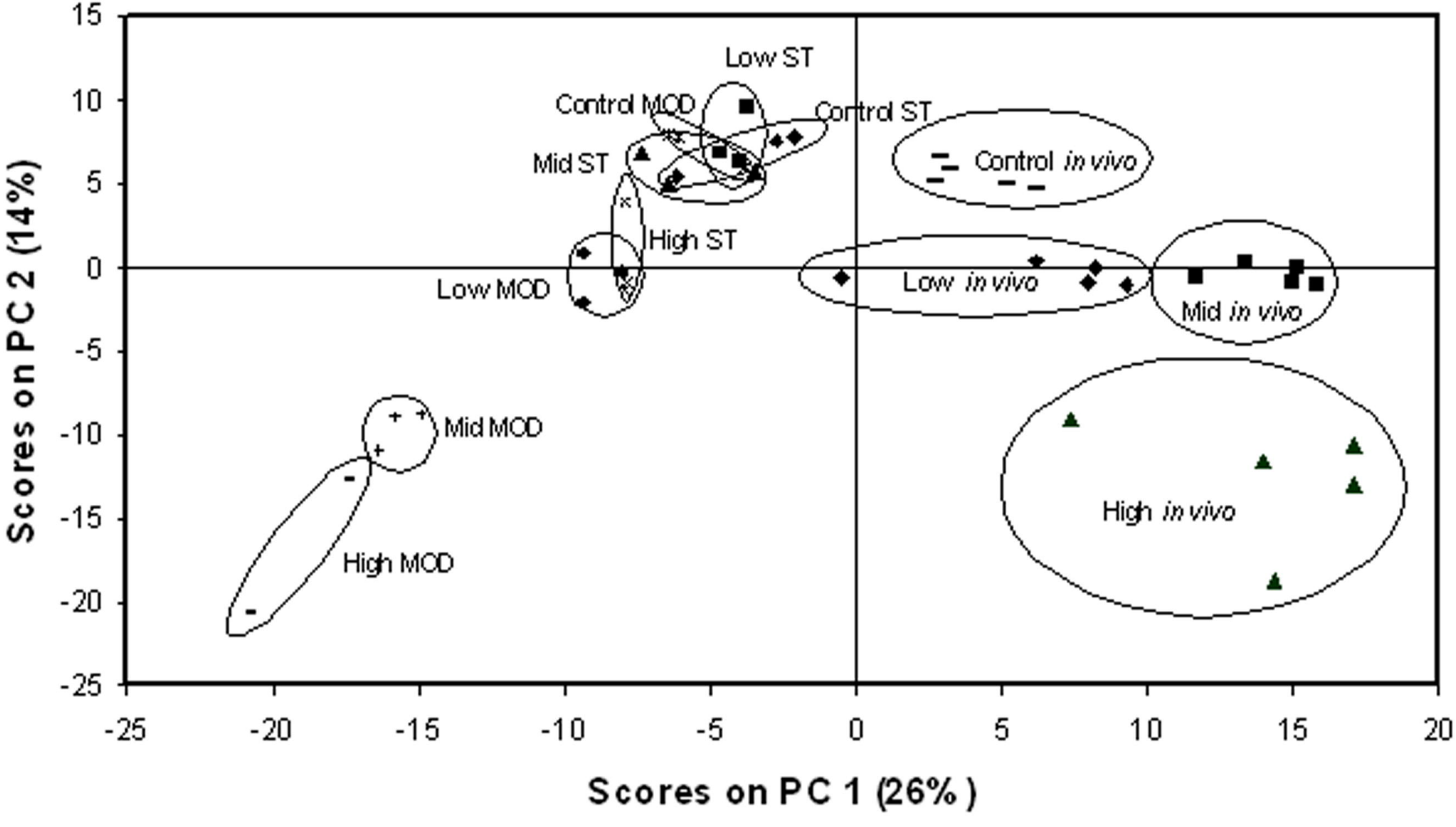
Low ST
Control MOD
Control ST
Mid ST
Control *in vivo*
High ST
Low MOD
Low *in vivo*
Mid *in vivo*
Mid MOD
High *in vivo*
High MOD
Scores on PC 2 (14%)
Scores on PC 1 (26%)
15
10
5
0
-5
-10
-15
-20
-25
-25
-20
-15
-10
-5
0
5
10
15
20

Fig. 3