Toxicogenomics

Applications of new functional genomics technologies in toxicology

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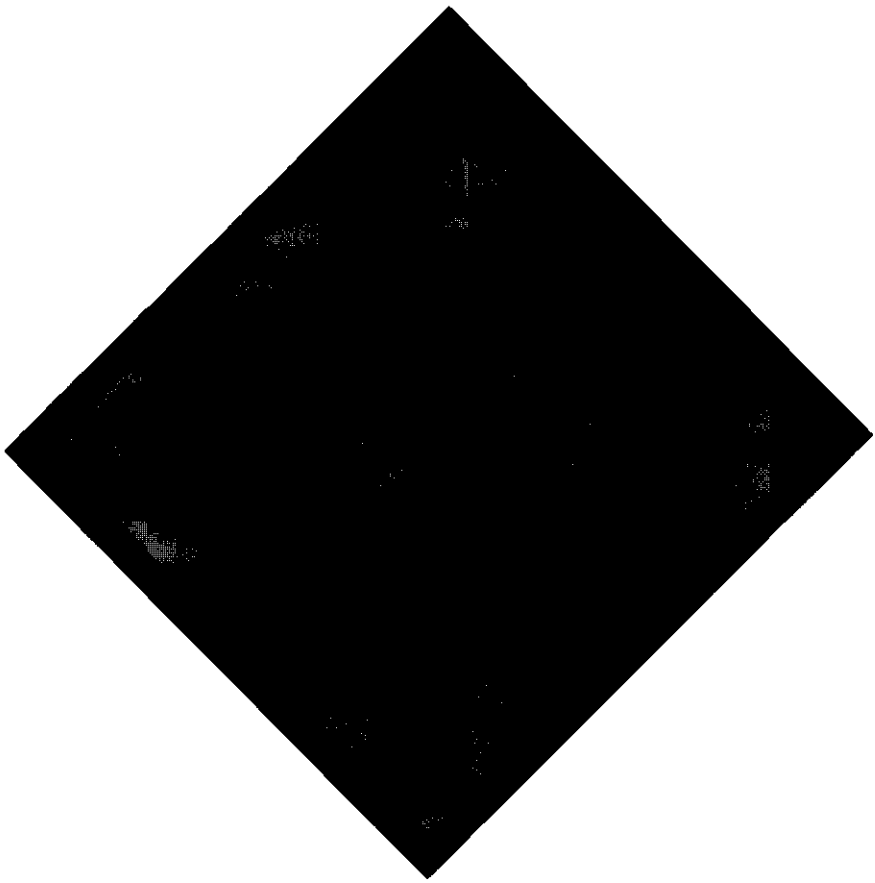

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I.1. Background

I.1.1 Toxicological risk assessment

Toxicology investigates adverse effects on organisms, induced by substances in their environment. To assess whether a substance forms a health risk, the potential hazards of the substance have to be identified. This involves empirical toxicity studies and investigation of the mechanism of toxic action (figure I.1) Substances in a mixture may cause unexpected effects (interactions). Extrapolations are needed to interpret short-term and high dose studies to real-life long term exposures to low doses. *Intra*species extrapolation accounts for differences in response between individuals. *Inter*species extrapolation is required to translate the results obtained in animal toxicity studies to the human situation. The factors are taken into account to determine an uncertainty factor, which is applied in combination with exposure levels to assess human health risk.

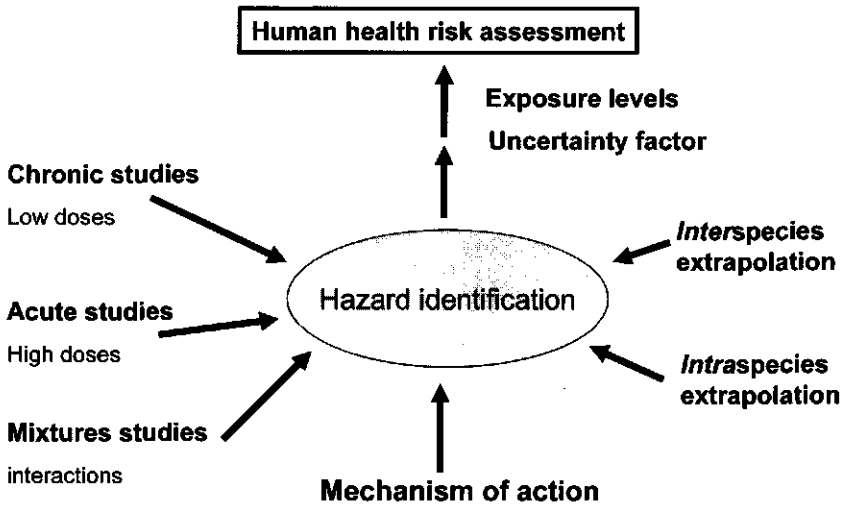

Figure I.1 *Schematic overview of human health risk assessment.*
The identification of hazard involves empirical testing in acute and chronic toxicity studies, and assessment of (unexpected) effects of mixtures. Mechanism of toxic action of the compound may be investigated, to enable *intra*- and *inter*species extrapolations. The factors are taken into account to determine an uncertainty factor, which is applied in combination with exposure levels to assess human health risk.

I.1.2 Current toxicological methods for hazard identification

To determine potential hazards of a substance, a set of toxicity studies has to be performed according to international standards (EC, OECD, FDA). In these studies, the acute and chronic toxicity of the compound will be established, based on a number of parameters, such as (histo)pathology, clinical chemistry and hematology. Pathology and histopathology form the basis of routine toxicity studies. Pathological examination may reveal changes in body and organ weights and macroscopic abnormalities. Histopathological techniques enable the microscopic examination of tissues, to detect toxic effects at the cellular level. Clinical chemistry, and hematology are used to determine changes in blood that correlate with

certain types of toxicity. Most of these parameters were established empirically. They are the result of aberrant cellular processes and late effects of toxicity, rather than components of mechanisms leading to toxicity. For instance, leakage of enzymes through disrupted cell membranes is observed in blood by clinical chemistry, and is clearly a secondary process in a late stage of cell death. Dependent on the complexity of the study, biochemical analyses of tissue samples and urine analysis may be required to provide complementary information about the compound under study. However, most of the routine toxicity studies determine adverse effects empirically and provide few insights in mechanisms of toxicity.
The development of small-scale molecular biological techniques enabled to determine the composition and expression of specific genes and proteins, relevant to mechanisms of toxicity. These methods (eg. RT-PCR, Northern and Western blotting, ELISA, see table I.1) are intended to study mechanisms of toxicity and interindividual or interspecies differences, at the molecular level. Specific molecular markers can distinguish different forms of toxicity within an organism or even within a tissue. Also, specific molecular endpoints may be detected early and at lower exposure levels compared to histopathology, clinical chemistry or hematology. However, these small-scale methods are applied only if a preconceived notion exists on the possible mode of action of the substance. Moreover, they will only reveal effects on single molecules.

I.1.3 New functional genomics technologies in toxicology

New technologies are being developed that could enable cell-wide and detailed analysis of mechanisms of toxicity, without the need of *a priori* knowledge on the mode of toxic action. As a result of the recent elucidation of complete genome sequences, functional genomics disciplines have emerged. These disciplines study the functions of the (complete) genome in relation to its composition, and measure gene expression (transcriptomics), protein levels (proteomics) or metabolite contents (metabolomics). Toxicogenomics can be defined as the application of the functional genomics technologies in toxicology. Toxicogenomics studies the adverse effects of xenobiotic substances in relation to the structure and activity of the genome [9]. The different disciplines and technologies are described in detail at the end of this chapter. First, applications of the functional genomics technologies in toxicology will be discussed.

I.2 Applications of functional genomics technologies in toxicology

Toxicology is likely to benefit from the new functional genomics technologies, as discussed in various publications [10-20]. Toxicogenomics methods may enhance health risk assessment in various ways. Detailed investigation of mechanisms of toxicity may enable to identify critical effects and the sequence of events at the molecular level (section I.2.1). Adverse effects in (animal) toxicity studies could be detected earlier and more sensitively than with the current methods (section I.2.2). Toxicogenomics results may enable more accurate extrapolations and the identification or even prediction of interactions upon exposure to mixtures (sections I.2.3, I.2.4). Eventually, toxicogenomics could enable to determine or predict the implications of the (individual's) genome composition for the toxic effects caused by substances. *In vitro* models in combination with toxicogenomics methods might allow to reduce, refine and possibly even replace *in vivo* testing (section I.2.5 ,I.2.6).

I.2.1 Studying mechanisms of toxic action

Changes in protein and gene expression play a role in signal transduction mechanisms, metabolic pathways and protective responses in a cell. In reaction to an insult, the changes at the protein and gene expression level in the cell precede the response at the tissue or even organism level. Thus, mechanisms leading to pathologically observed endpoints may be recognised through observation of changes in expression of genes and proteins. The functional genomics technologies could be useful to elucidate the critical steps and the sequence of events in the development of toxicity after exposure to toxic substances.

A toxic compound, by itself, or via reactive metabolites after bioactivation, elicits a cellular response upon interaction with a target molecule. This target may be a receptor molecule that initiates a response that involves many other molecules. The primary target of the toxicant might not always be identified directly through changes in gene or protein expression. However, the downstream cascade of effects may help to find the initial targets at the origin of the response. As thousands of molecules are investigated simultaneously, the chance of identifying (unexpected) molecules affected by the xenobiotic is greatly improved. This might also enable to recognise adverse side-effects of pharmaceutical compounds.

Pathological endpoints such as hepatic necrosis result from a spectrum of events, often non-specific processes like membrane damage, ATP depletion and apoptosis. These processes could be associated with characteristic gene expression changes. Model compounds known to specifically elicit one of these events could be helpful to identify molecular changes corresponding to these events. To this date, several toxicogenomics studies were performed with model compounds that induced different effects in liver, such as hepatic necrosis, biotransformation enzyme induction and peroxisome proliferation [12]; [21-27]. Metabolomics was used, for instance, to analyse toxicity through metabolites in liver, plasma and urine of rats treated with model hepatotoxicants and nephrotoxicants [18]; [28-33].

I.2.2. Identification of toxicity markers

Toxicogenomics methods measure thousands of genes, proteins or metabolites. Therefore, it is expected that sensitive and specific markers will be identified that can detect or even predict pathological abnormalities at early stages, and after exposure to low dose levels. Toxicogenomics patterns of changes will be more efficient than single molecule markers to discern slightly different mechanisms that lead to similar adverse effects. Subtle changes in levels of many molecules are likely to precede the conventional symptoms of toxicity. A cell-wide pattern of gene or protein expression, in analogy to a fingerprint, can be used to early

discern a healthy cell from a cell with slight abnormalities. Gene expression profiles have been applied successfully to discriminate samples exposed to different classes of toxicants [22];[34];[35]. Transcriptomics was also used to classify blind-coded liver samples, according to the class of toxicant to which the rats were exposed [36]. Proteomics methods with 2D-gelelectrophoresis were applied a.o., to identify biomarkers of acute nephrotoxicity induced by puromycin aminonucleoside [37]. Metabolomics has been used to classify urine samples of rats treated with either a liver or a kidney toxicant [31];[18]. Urine profiles were analyzed in time upon single dosage of ANIT, galactosamine and butylated hydroxytoluene [32]. Time-related differences in metabolite contents were related to the stage of the lesions, and specific changes in metabolite levels were identified for each compound.
Metabolite profiling can also be used to assess levels of exposure or confirm successful dosing by measuring the (metabolites of) toxicants or drugs in blood plasma or urine. A great advantage is that samples obtained by non-invasive methods (urine, blood, saliva) can be used for measurements. All functional genomics methods can be applied to identify new molecules as markers of toxicity, while also the complete patterns of changes may be characteristic and robust markers of adverse effects.

I.2.3 Interspecies and intraspecies extrapolation

The extrapolation of results from toxicity studies to account for *inter*species and *intra*species differences increases the uncertainties for human health risk assessment. Toxicogenomics methods may reduce the uncertainty by providing detailed insight in molecular mechanisms underlying the toxic endpoints. While these (physiological) endpoints might differ, the underlying molecular mechanisms may be conserved to a much higher extent between species. Laboratory animals and humans, are highly similar at the molecular level. The majority of the genes found in humans are also present in organisms like rat or mouse, and genomes of man and rodents exhibit more than 90% similarity. This is of great benefit for the extrapolation of results of toxicity studies at the molecular level. Thus, so-called bridging effect markers may be developed, that can be used in extrapolation to humans of effects found in animals. Furthermore, the identification of critical steps in molecular mechanisms that to explain empirically observed *intra*- and *inter*species differences in response to toxicants might be feasible with toxicogenomics methods. Because less than 0.1% of the genome differs between individual persons, it should be possible to characterise many of the small differences ("SNP mapping") with toxicogenomics (or toxicogenetics) methods, and identify genetic differences responsible for susceptibility to toxicants [38]. In summary, toxicogenomics has the potential to improve extrapolations by providing more insight in mechanisms at the molecular level.

I.2.4 Exposure to mixtures of compounds

Harmful effects can be expected to be caused by more than one substance in complex mixtures, such as environmental pollutants or even nutrition. A thorough analysis of the toxicity of mixtures is technically difficult. In general, toxicity data of studies with single compound exposures are used to predict effects of exposure to mixtures of compounds. However, sometimes effects are observed that deviate from the predictions. These interaction effects may be synergistic, or antagonistic. In order to assess interaction effects of compounds, large studies have to be designed including exposures to individual chemicals as well as to combinations at different dose levels. Currently, the identification of toxic interaction effects is limited by the capacity of the available markers of toxicity to distinguish

and quantify effects after treatment with mixtures in comparison with single compounds. Functional genomics technologies allow the monitoring of thousands of effects and will enhance the discrimination of exposure groups based on observed effects. Mechanisms of the combinatorial effects can be studied and possibly predicted at the molecular level. When different substances in the mixture affect similar molecules or pathways, enhanced effects might be expected. When changes are provoked in different biological pathways, interference by the compounds in the mixture would not be expected. Because of the complexity of an organism, secondary effects of one substance could interfere with the mechanism of toxicity of another substance. For instance, a depletion of the intracellular antioxidant defense system by reactive metabolites of a compound could lower the threshold for damage by another xenobiotic. In conclusion, toxicogenomics methods will facilitate the assessment and prediction of putative harmful interaction effects upon exposure to mixtures of substances.

I.2.5. Toxicogenomics in combination with in vitro testing

Toxicogenomics might be able to support the use of *in vitro* models for various reasons. Although pathological endpoints of toxicity can not be identified *in vitro*, the preceding or underlying molecular mechanisms eventually leading to these effects can be analysed. Both *in vitro* and *in vivo*, the same sets of thousands of genes, or proteins can be measured and directly compared. Thus, toxicogenomics could enhance extrapolations of results from *in vitro* experiments to *in vivo* circumstances Toxicogenomics may enable better identification and discrimination of responses in the *in vitro* models, compared to the currently available methods. In *in vitro* experiments, the circumstances, such as exposure conditions, can be defined much better than in the *in vivo* situation. Metabolism of parent compounds to reactive metabolites can be followed, and metabolites are accessible for analysis, eg. with a metabolomics approach. Toxicogenomics methods make it possible to evaluate and compare multiple aspects of the *in vitro* models with the *in vivo* situation. This will drive the development of models that better mimic the real situation.

Toxicogenomics has great potential to facilitate the recognition of toxic properties in the early stages of screening of candidate drugs. Few of the many newly synthesised candidate drugs are evaluated in time and cost intensive assessments of efficacy and toxicity. Potential signs of toxicity in *in vitro* models may therefore provide a useful criterion for early selection or prioritising of drug candidates. The gene or protein expression profiles induced by the compounds could, like a fingerprint, be used to classify compounds according to toxic potency, mechanism of action and target organ. A novel compound could be classified as putatively toxic if it induces the same transcriptional changes as known toxicants ("guilt by association"). Several studies have shown the feasibility of this concept in animal experiments [39] [22], while a few also applied this to *in vitro* model systems with cultured hepatocytes [34]; [40-42]. Concluding, toxicogenomics can support the use and the further development of *in vitro* models, and could save resources when applied for early *in vitro* screening of toxicity.

I.2.6 Reduction, refinement and replacement of animals testing

The recognition of toxicity with toxicogenomics-based markers after short-term exposure could reduce long-term animal exposures. In particular for assessment of carcinogenic properties of compounds, a considerable amelioration is expected. If toxicogenomics methods enable to test toxicity of compounds at lower dose levels, this will reduce the

discomfort of the animals. Possibly, less animals are needed to determine mechanisms of toxicity of a compound when functional genomics technologies are fully exploited. Detailed insights in mechanisms can be used to refine toxicity tests and select appropriate models for further studies. As described earlier, toxicogenomics might enhance identification of toxicity in alternative *in vitro* models rather than in live animals. It may enhance the extrapolation of test results for human health risk assessment. Thus, toxicogenomics facilitates the reduction and refinement and perhaps eventually the replacement of animal testing, especially when applied in combination with predictive *in vitro* models.

I.3 Functional genomics technologies

Functional genomics technologies measure molecular responses in an organism in a cell-wide manner. The description of the full genome sequence of human and model organisms became available recently, and knowledge about proteins and metabolites in cells is increasing rapidly. Genomics is the sub-discipline of biology that studies the genome through analysis of the nucleotide sequence, the genome structure and its composition. Transcriptomics, proteomics and metabolomics determine the levels of gene transcripts, proteins or metabolites, respectively. Figure I.2 displays the relation between DNA, gene expression, protein production and activity (towards metabolites) in the cell. A schematic representation of the different cellular molecules and the novel technologies to study these is provided in table I.1. The disciplines and the corresponding technologies, as well as the bioinformatics methods for data interpretation are discussed in detail in the following sections.

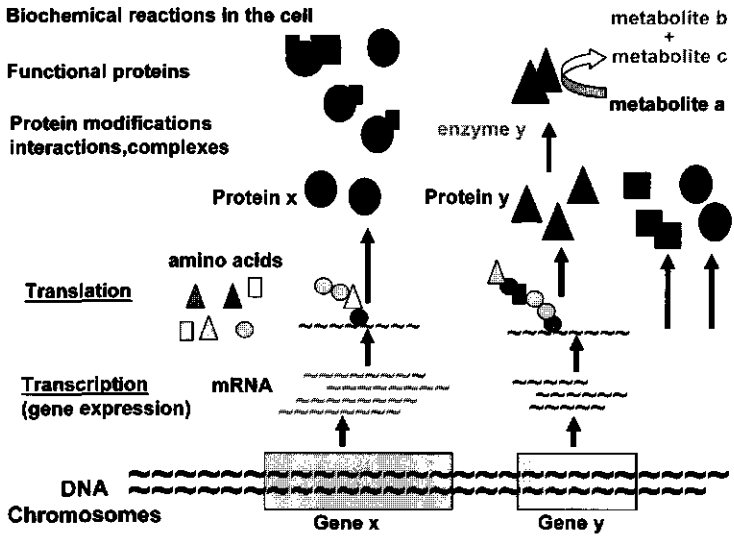

Figure I.2 *DNA, gene expression, protein production and activity*
Chromosomes contain the (>30000) genes. When genes are expressed, complementary mRNA is transcribed from DNA. Messengers (mRNA) transfer the information for correct synthesis of proteins from free amino acids (translation). Chains of amino acids (peptides) are shaped into proteins, which may require post-translational modifications to be functional in (biochemical) processes in the cell. Functional proteins, for example, are enzymes that catalyse conversions of metabolites.

Table I.1 *Functional genomics technologies*
The functional genomics disciplines and (small and large-scale) technologies that are currently used to study DNA, gene expression, proteins, and metabolites.

Subject of study	Discipline	Technologies
DNA composition of genome, chromosomes and genes	**Genomics**	Automated sequencing, SNP detection, array CGH, FISH, Southern blotting, AFLP
Gene expression through levels of mRNAs (transcripts)	**Transcriptomics**	DNA microarrays (cDNA or oligonucleotides) branched DNA assay, RT-PCR, SAGE, Northern blotting
Proteins protein contents of tissues, cells, and body fluids	**Proteomics**	2D-gel electrophoresis, Mass spectrometry, SELDI, ICAT, protein arrays, ELISA, Western blotting, immunodetection
Metabolites (small molecules)	**Metabolomics**	NMR spectrometry, GC-MS, MS-MS,

I.3.1 Genomics

The genetic material contains the basic information needed for the production of all cellular proteins. Knowing the composition of this genetic material is a major step towards the elucidation of molecular mechanisms. Information about the organisation of genes and transcriptional regulatory elements on chromosomes contributes to the understanding of gene-functions and their role in specific processes. In comparative genomics, complete genomes from different organisms are compared to investigate shared as well as unique cellular mechanisms and physiological properties of different species.

Genotyping involves the identification of (small) modifications in individual genomes. Single nucleotide polymorphisms (SNPs) are responsible for many *inter*individual differences and for genetic diseases, and may be of great importance for toxicology. Less than 0.1% of the genome differs between individual persons, but these small genetic differences may account for large effects. Inherited diseases, but also large differences in susceptibility to xenobiotic toxicants may result from these small polymorphisms [38]. For example, if a drug metabolising enzyme is slightly altered, its activity could be highly compromised. Therefore, metabolism of a xenobiotic substance will be altered, with consequences for the toxicity found in the organism. Genetic differences may also influence gene expression levels. Analysis of the composition of the genome and *inter*individual differences will facilitate assessment of susceptibility to toxic substances, valuable for health risk assessment.

I.3.2. Transcriptomics

Many cellular processes are controlled at the level of gene expression. Determination of changes in gene expression, by measurement of the mRNA levels, is used to predict changes in protein levels and activity. Single-strand DNA or RNA has the capacity to specifically hybridise to its complementary strand. This capacity is used in several methods that determine mRNA levels of gene of interest. Specific detection with radiolabelled nucleotides is called Northern blotting. The development of the reverse transcription polymerase chain reaction (RT-PCR) enabled extremely sensitive, semi-quantitative detection of specific mRNA in a sample. Specific mRNA levels can be quantified in comparison to a constitutively expressed, "housekeeping" gene with quantitative real time PCR. Northern blotting and PCR techniques are very labour-intensive and not suitable for scale-up. Serial analysis of gene expression (SAGE) is a scaled-up method that determines the sequence of hundreds of mRNA fragments, and counts the specific mRNAs in the sample. Recently, DNA microarrays [43] or DNA chips [44] were developed as a large-scale method for gene expression measurement, and the technologies have been described extensively [45-50]. Like in Northern blots, the capacity of single-strand DNA and RNA to specifically hybridise is used to sort the mRNAs and determine gene expression levels. However, instead of one gene at the time, single-strand cDNA or oligonucleotide molecules for thousands of different genes are deposited in a fixed spot on a surface (e.g. glass slide, plastic, nylon membrane). The microarrays can be custom-made in the laboratory, or obtained from commercial providers (eg. affymetrix, agilent, incyte, sigma, clonetech). Microarrays take advantage of the availability of collections (libraries) of gene fragments with a known sequence and with annotation concerning their (putative) function in the cell. Microarrays can contain DNA fragments for which a function has yet to be determined. The process of microarray-based measurement of gene expression is depicted in figure I.3a, and an image of a section of a hybridised microarray is shown in figure I.3b.

By hybridisation of the cDNA micorarray with a pool of isolated mRNAs, each specific mRNA will only hybridise with the cDNA in the spot containing the complementary cDNA for this specific gene. The amount of cDNA hybridised in each spot can be detected by measurement of fluorescence that was incorporated in the sample. In practice, two samples are hybridised together on the cDNA microarray, where the test sample is labelled with one type of fluorophore (e.g. green) and the reference or control sample is labelled with another type (e.g. red). Quantification of both types of fluorescence enables the determination of a ratio of expression for each gene in the test sample with respect to the control sample. Data analysis of DNA microarray experiments will be described in detail in section I.3.5 and figure I.6. The majority of the thousands of genes will not show differences in expression levels when diseased or treated samples are compared to controls. The gene expression levels that are induced or reduced provide a wealth of information on cellular mechanisms that are affected at the gene expression level by the disease or treatment.

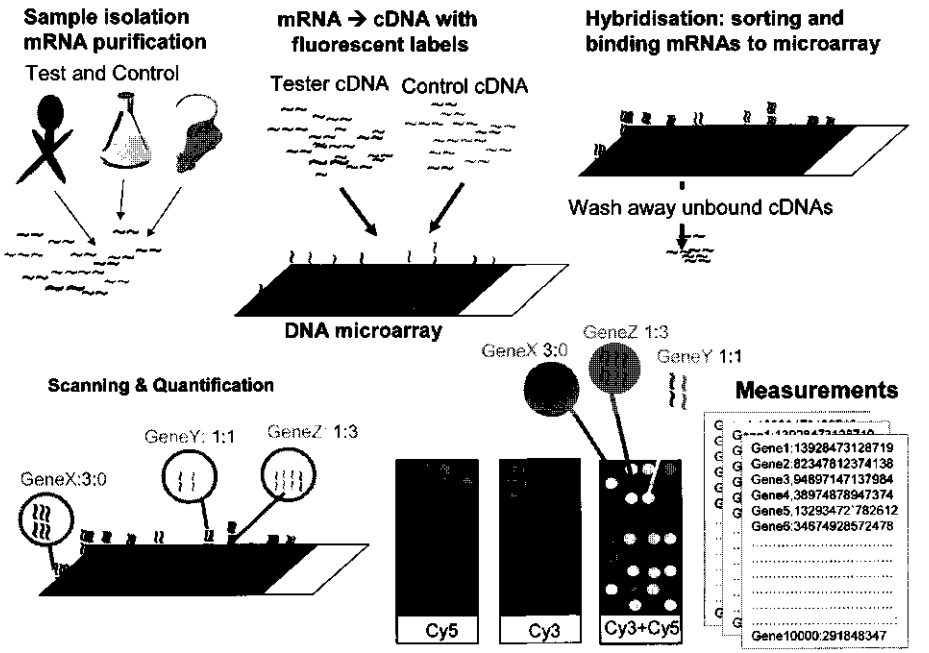

Figure I.3.a *Transcriptomics method using DNA microarrays*
The process of transcriptomics using DNA microarrays to measure differential gene expression in a test compared to a control sample. Isolated mRNA of a test and control sample is labelled with different fluorescent labels and hybridised to the microarray. Thus, expression levels can be quantified from the fluorescence in each spot representing a different gene. Ratios of intensities of the two types of fluorescence can be used to determine relative gene expression levels in test and control samples.

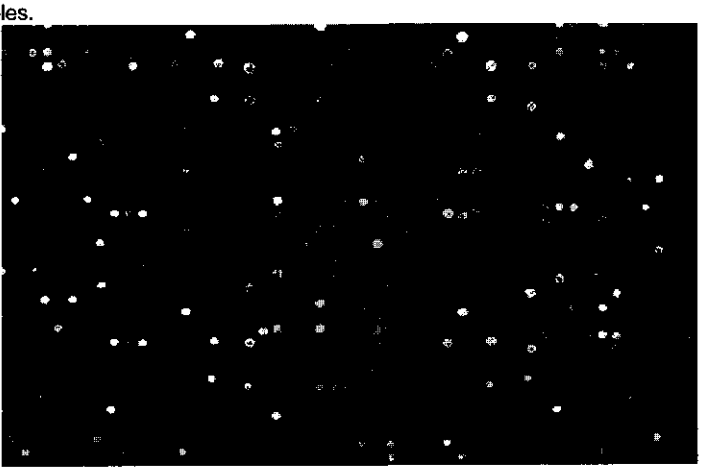

Figure I.3.b *Fragment of a DNA microarray*
Fragment of a scanned rat oligonucleotide DNA microarray containing around 5000 different gene fragments, spotted in duplicate. This DNA microarray was hybridised with RNA from a control rat liver and a sample from a rat treated with a liver toxicant.

I.3.3 Proteomics

Not all changes in cellular mechanisms can be measured at the gene expression level. Protein modification or sub-cellular redistribution of proteins provide an organism with rapid mechanisms to respond to stimuli, without the need for gene expression. Proteomics technologies measure the thousands of proteins in a cell or body fluid. Posttranslational modifications and protein complexes can be visualised. Because proteins are the actors in cellular reactions, rather than gene transcripts, proteomics is likely more relevant than transcriptomics. However, the measurement of the proteome is more complex, because the separation of the proteins in a cell or sample is a difficult task, as all the proteins have different properties (mass, iso-electric point, solubility, stability, etc.).

Initial proteomics methods combined two-dimensional gel electrophoresis with automated image analysis software and mass spectrometry [51-55]. This process is schematically drawn in figure I.4. The proteins in a sample are first separated according to their iso-electric point using a pH gradient and secondly separated based on mass by standard electrophoresis. Separated protein spots are visualised with fluorescent or silver staining. Gels are scanned and dedicated software quantifies the spot volumes. Sometimes, protein spots can be putatively identified by matching to a reference gel in (internet) databases. Spots of interest can be isolated, and identified by mass spectrometry after fragmentation (e.g. digestion with trypsin). The pattern of peptide fragment with very specific masses is determined by (matrix assisted laser desorption ionisation time-of flight) mass spectrometry (MALDI-TOF-MS)[56-57]. The protein is identified by matching the pattern to predicted patterns of all known proteins in a database. Peptide sequencing by MS/MS techniques can further confirm identities.

More recent proteomics methods applied protein antibody arrays, comparable to DNA microarrays [58];[59]. A major drawback is that binding affinity varies largely between proteins. Optimised conditions for binding are only applicable for part of the proteins and suboptimal binding will bias the results. Methods that exploit these differences in binding affinity to sort proteins include ICAT (isotope-encoded affinity tags, [60]) and SELDI (surface-enhanced laser desorption / ionisation [61], [62]). Different surface chemistries are used to selectively isolate proteins and analyse them by mass spectrometry.

In summary, proteomics techniques provide a wealth of detailed information about protein contents and protein modifications in cells, tissues or body fluids, that may help to better understand molecular processes, for instance in response to toxic compounds.

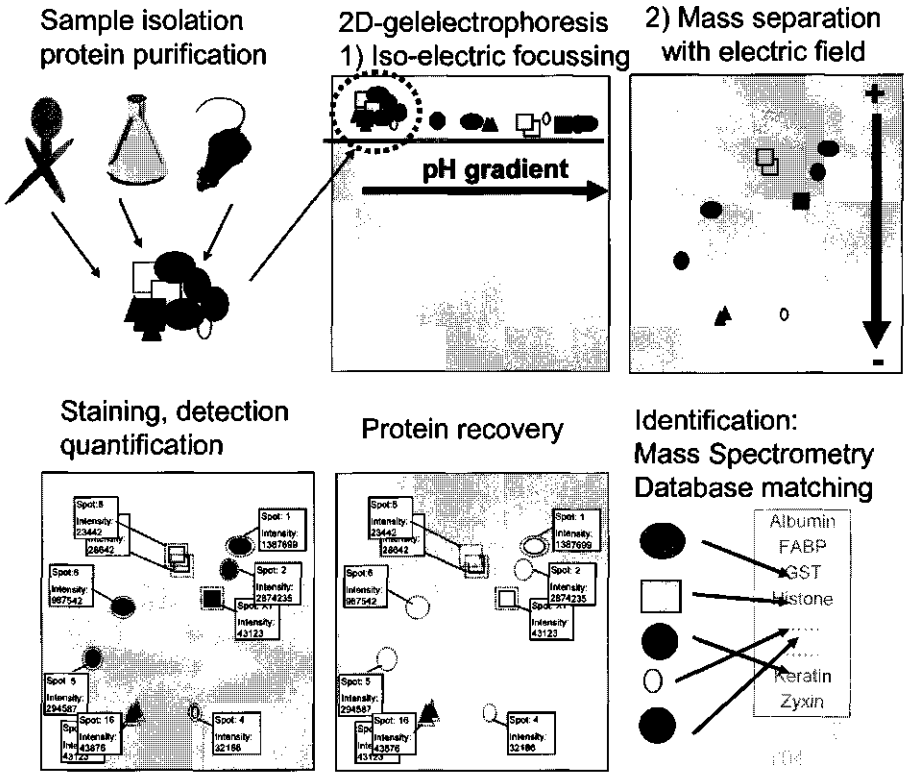

Figure I.4. *The proteomics method with 2D-gel electrophoresis*
Protein samples are separated using a two step 2D-gelelectrophoresis. Spots are quantified using automated gel analysis. After excision of the protein spot from the gel, tha material can be analysed with mass spectrometry for protein identification, using a reference database.

I.3.4 Metabolomics (metabolite profiling)

Metabolomics methods measure the levels of metabolite (small molecules) in the cell and in extracellular fluids, which may reflect changes in cellular processes. The dynamics of biochemical reactions in the cell can be studied by monitoring the (dis)appearance of reaction products. Processes like metabolism and biotransformation of xenobiotics can be followed in cells or plasma, and excreted metabolites can be determined in the urine. Various applications of metabolomics in toxicology have been reported and reviewed [18]; [63]; [64]; [65]. Metabolomics methods include gas chromatography and mass spectrometry (GC/MS), that allow the measurement of low concentrations of individual components. Proton nuclear magnetic resonance (1H-NMR) spectroscopy is an attractive approach to simultaneously quantify a wide range of metabolites without extensive sample preparation. A spectrum is obtained with resonance signals characteristic for all small biomolecules. Thus, a metabolic fingerprint may be used to characterise biological samples. Individual signals can be quantified and identified in using reference spectra, if available in databases. NMR spectra of biological fluids are very complex due to the mixture of numerous metabolites. Variations

between spectra can often not be recognised by eye. In order to compare NMR spectra and thereby maximise the power of the subsequent data analysis, a partial linear fit algorithm adjusts minor shifts in the spectra while maintaining the resolution. To find significant differences in the spectra, multivariate statistical data analysis is applied. This enables to explore recurrent patterns in multiple NMR spectra. A factor spectrum can be used to identify metabolite NMR peaks that differ between samples. An illustrative factor spectrum of NMR profiles of plasma of vehicle controls and rats after exposure to a hepatotoxicant is shown in figure I.5. Signals (metabolites) in the complex and large data sets can be correlated to a variable of interest, such as toxicity status. A combination of analytical techniques is desirable for an exhaustive analysis of a complex mixture of metabolites. A major advantage of metabolomics (for toxicology) is that non-invasive methods can be used to collect samples from blood (plasma), urine or other body fluids, which allows applications in human and animal experiments.

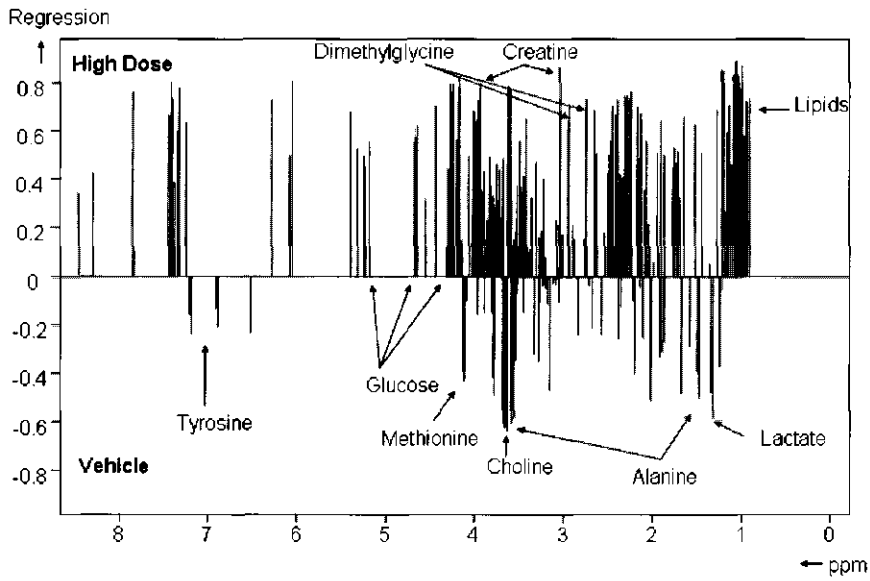

Figure I.5 *NMR Factor spectrum of urine metabolomics analysis*
Factor spectrum constructed using multivariate statistical analysis that compares average NMR spectra of blood plasma of rats. In this case, spectra obtained 6 hours after treatment with a high dose of liver toxicant are compared to vehicle controls.

I.3.5 Data processing and bioinformatics

The handling of raw data from large-scale functional genomics experiments requires powerful data processing equipment and algorithms (bioinformatics). The steps in pre-processing of raw data to clean data and the options for data mining and interpretation are depicted in figure I.6. Information that describes the experimental conditions should be automatically stored (e.g. in a Laboratory Information Management System (LIMS)) and organised in a searchable manner. Systematic bias originating from various technical sources should be corrected. A signal-to-noise threshold should exclude weak and unreliable signals from further interpretation. Standardisation, normalisation or scaling are methods that are applied to transform data sets in order to be able to compare the measurements within studies, but also between studies. After raw data processing, secondary results files are obtained that should be stored along with the description of the method of data analysis, preferably according to internationally established MIAME requirements (Minimal Information About Microarray Experiments). The clean data files are the basis for the interpretation of the results, described in the next section.

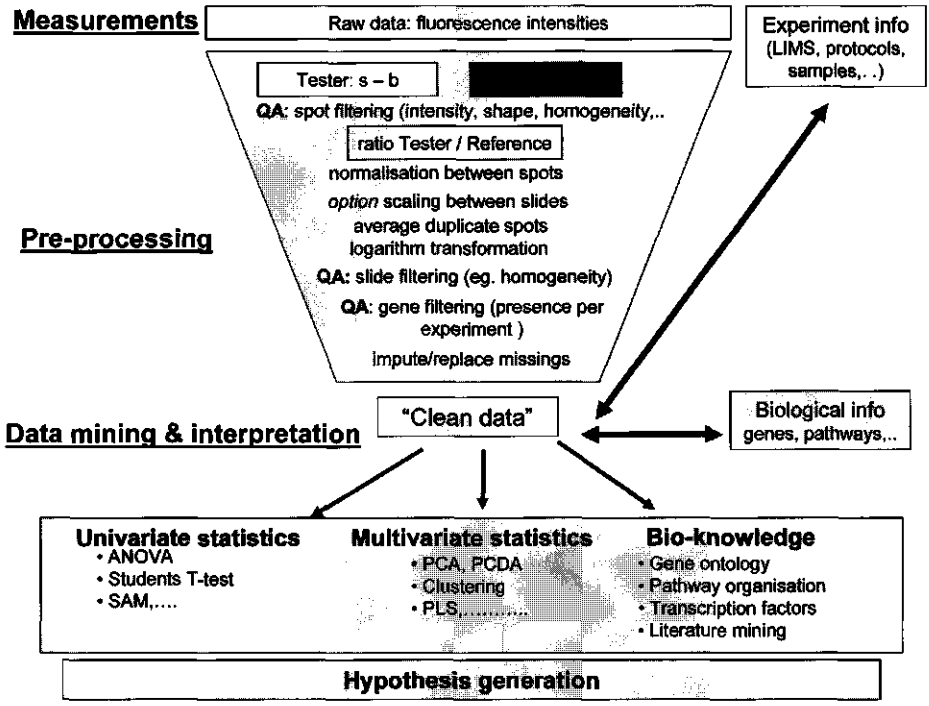

Figure I.6 *Transcriptomics data pre-processing and interpretation*
The steps in pre-processing of raw data to clean data and the options for data mining and interpretation are depicted schematically (according to in-house procedures at TNO).
QA indicates quality assessments at various stages in the analysis. After the raw data (fluorescence intensities) pre-processing into "clean data" these are linked to experimental and biological information. At this stage, the results are ready for data mining, biological interpretation and hypothesis generation.

I.3.6 Biological interpretation and hypothesis generation

The challenge of functional genomics methods is to turn the large data sets with relatively high amounts of noise and without obvious biological meaning into relevant findings. It is not feasible to analyse data per gene, protein or metabolite. Moreover, this would result in a great loss of the information that resides in the coherence of the data. The relationships between genes or proteins, and their changes under specific conditions, are probably the most valuable insights obtained in functional genomics studies. To assess relationships between molecules, the large datasets have to be structured and organised, also called data mining. Multivariate statistical techniques and the (automated) integration of measurements with pre-existing knowledge can facilitate data-mining.

Statistical techniques for data mining

Toxicogenomics data may be analysed with univariate and/or multivariate statistical techniques. Univariate statistical methods (eg. ANOVA, Student's T-tests) are efficient and typically used to determine significance of changes in a single parameter under study. A variety of multivariate statistical techniques can be applied to structure large datasets. Different methods of functional genomics data analysis and interpretation are reviewed in detail [66]; [67]; [68]. Mathematical clustering algorithms like hierarchical clustering, K-means clustering and self-organising maps calculate a measure of similarity between levels of gene or proteins in profiles. Clustering creates subsets of molecules that behave similar to each other and enables to select molecules with biologically relevant characteristics, out of the thousands of genes, proteins or metabolites. Unsupervised methods such as principal component analysis (PCA) determine intrinsic structure within data sets, without prior knowledge. PCA can be used to calculate a measure of similarity between large datasets, such as NMR spectra or DNA microarray measurements. Supervised methods such as partial least squares (PLS) and principal component discriminant analysis (PCDA) use additional information such as biochemical, histopathological or clinical data to optimise the discrimination between samples. These methods allow to identify the molecules in the large datasets that most contribute to differences between the samples, for instance induced by a toxic treatment.

Pre-existing knowledge for data mining

Structuring of large datasets can be achieved with the use of pre-existing knowledge about biological processes. The vast expansion of the world-wide web proved to be crucial for the rapid developments of functional genomics and bioinformatics. Global sharing of biological data and information enabled to link previously scattered and isolated information. Relying and building on the complete DNA sequence of the human genome, many sources of biological information on genes and proteins and interaction with the environment have become available. These sources each provide information concerning annotation of genes and proteins, gene functions, structures and other properties of proteins, biochemical processes and relations of genes and proteins with physiological changes, eg. a diseases (table I.2). Uniform and standardised names and functional categories are assigned to each gene, protein and metabolite. This process, called ontology, is essential for correct exchange of results.

The development of methods for automated literature searching and interpretation holds great promise. This would allow to more easily utilise the data gathered in the past.

By categorising the molecules according to biological processes, rather than finding changes in one enzyme, changes in entire biochemical pathway can be identified in response to applied conditions. Textbooks and schematic drawings of pathways can be used to explain individual changes in the context of pathways. This is facilitated by conversion of pathway schemes into electronic versions, with extensive annotation and flexibility. Computer software is being developed to visualise effects, for instance changes in gene expression, in the context of the biological process of interest. Thus applied, genomics experiments can provide insights in entire changes in pathways and processes, besides changes in individual molecules.

After identification of the molecules involved in the response to the environmental conditions, only these molecules can be analysed in further, dedicated, studies. It is important to realise that changes at the molecular level are biologically relevant only if they are associated with changes in the physiology of the organism. In toxicology, for instance, reversible changes are referred to as adaptations, while only irreversible effects are considered to be adverse and, therefore, pose a putative health risk. The effects observed with the functional genomics methods most often generate new hypotheses that have to be confirmed with other techniques, and fitted into the context of physiological effects. In toxicology, this means that results from toxicogenomics studies have to be integrated with effects seen with histopathology or other conventional methods. The many possible applications of functional genomics methods will only be fully deployed when these methods are integrated with conventional techniques, to allow an iterative process of hypothesis generation and confirmation.

Table I.2 *Selection of worldwide web sources of biological information*

Provider	Information type	U.R.L.
EBI: European Bioinformatics Institute	various services	www.ebi.ac.uk
NCBI: National Centre for Biotechnology Information	various services	www.ncbi.nlm.nih.gov
Swissprot + Boehringer maps	various services (proteins)	www.expasy.org
BioASP, The Netherlands	various services	www.bioasp.com
Gene ontology consortium	Gene ontology (annotation)	www.geneontology.org
RGD: Rat Genome database	rat gene database	rgd.mcw.edu
MGD: Mouse genome database	mouse gene database	www.informatics.jax.org
Source (Stanford university)	gene information	source.stanford.edu
GeneCards	gene information	bioinfo.weizmann.ac.il/cards
KEGG pathway maps	biochemical pathways	www.genome.ad.jp/kegg
GenMapp	visualisation of pathways	www.genmapp.org
Biocarta	biochemical pathways	www.biocarta.com

I.4 Objectives of this thesis

The studies described in this thesis were performed to demonstrate the potential value of toxicogenomics, the integration of functional genomics methods in toxicology. Many possible applications of toxicogenomics were described earlier in this introduction, and this thesis primarily demonstrated the use of toxicogenomics to:

a) investigate mechanisms of toxicity (see I.2.1)

The aim was to characterise gene and protein expression changes in relation to pathological abnormalities, after exposure to (potentially) toxic compounds. The studies concentrated on analysis of toxicity in rat liver, which is probably the most critical and best studied target organ of toxicity. Also, the liver plays an important role in detoxification, metabolic activation and systemic distribution of many (xenobiotic) substances.

b) obtain new markers of toxicity (see I.2.2)

The studies aimed to discover new markers of hepatotoxicity with transcriptomics, proteomics and metabolomics methods. These markers should enable early identification of adverse effects, at exposure to lower dose levels in comparison to the current toxicity markers.

c) assess effects of mixtures of toxic compounds (see I.2.4)

The objective was to use gene expression profiles to assess and explain putative joint effects and interactions upon exposure to mixtures of chemicals.

Furthermore, technical aspects of toxicogenomics methods were evaluated. The reproducibility and sensitivity were determined for transcriptomics, proteomics and metabolomics methods, in relation to conventional methods in toxicology. The relationship between transcriptomics, proteomics and metabolomics was investigated in toxicological applications. The integration of these different methods in toxicogenomics could be crucial for the understanding of the sequence of events and the critical steps in the mechanisms of toxicity at the molecular level.

I.5 Outline of this thesis

This thesis describes the toxicogenomics analyses of the response in rats after exposure to bromobenzene, benzene and trichloroethylene and mixtures of benzene and trichloroethylene. **Chapter I** introduces possible applications and the different technologies used in toxicogenomics. **Chapter II** describes the analysis of toxicity at the level of gene and protein expression in liver. The effects of bromobenzene, a well-known model hepatotoxicant, were studied in rat liver with transcriptomics and proteomics, 24 hours after single dosage. The methods were evaluated and compared and toxicological interpretation of the results corroborated existing, and raised new hypotheses regarding the mechanisms of hepatotoxicity of bromobenzene. The toxicity principle that the dose of a compound determines its effects, prompted us to establish how the bromobenzene-induced changes at the gene expression level were dependent on the dose. **Chapter III** describes hepatotoxicity induced by different doses of bromobenzene at the transcriptome level. The time dependency of gene expression changes was determined 6, 24 and 48 hours after dosage. The results were related to routine toxicity parameters. The transcriptomics data from this study were analysed in combination with nuclear magnetic resonance (NMR) measurements of metabolite profiles in urine and blood plasma samples of the same study, as described in **chapter IV**. The aim was to determine whether a combined analysis of the patterns of hepatic gene expression and urine or plasma metabolite profiles would improve the detection of toxicity. We also investigated whether new insights could be obtained in the molecular mechanisms leading to chemically induced hepatic necrosis. Multivariate statistical methods were applied in order to fully exploit the large amounts of data and facilitate biological interpretations.

Previous chapters describe the effects of a model toxicant that induces obvious signs of liver damage within days. In **chapter V and VI,** effects at the liver gene expression level are reported after exposure for 28 days to different concentrations of widely used chemicals, benzene and trichloroethylene (TCE). The metabolism of both TCE and benzene primarily takes place in liver and determines the toxic effects in the target organs. For benzene, these are the blood and bone marrow, and exposure can ultimately provoke leukemia. TCE is nephrotoxic and a suspected carcinogen. Both compounds are not known as hepatotoxicants, although exposure can lead to increased liver weights. Analysis of hepatic gene expression changes in response to benzene and TCE aimed to delineate the specificity of the effects induced by (potentially) hepatotoxic chemicals. Therefore, effects of benzene and TCE were compared to earlier effects of the well-known hepatotoxicants bromobenzene, described earlier in this thesis, and published data on acetaminophen [69]. In **Chapter VII,** toxic effects of mixtures of benzene and trichloroethylene in liver are analysed using transcriptomics. To our knowledge, this thesis presents the first approach to investigate combined action of mixtures of (toxic) compounds at the gene expression level. For this analysis, rats were exposed to benzene, trichloroethylene or to mixtures of these chemicals at different dose-levels. Gene expression profile changes were used in a multivariate statistical model to assess putative interaction effects. Expression changes relevant to the mechanisms of toxicity of both benzene and TCE were analysed in more detail. In **chapter VIII,** the findings of all studies are summarised. Technical aspects of the technologies, and possible applications of toxicogenomics are discussed using the results of the studies described in chapters II VII, and lastly future challenges are mentioned.

Chapter II

Toxicogenomics of bromobenzene hepatotoxicity: a combined transcriptomics and proteomics approach

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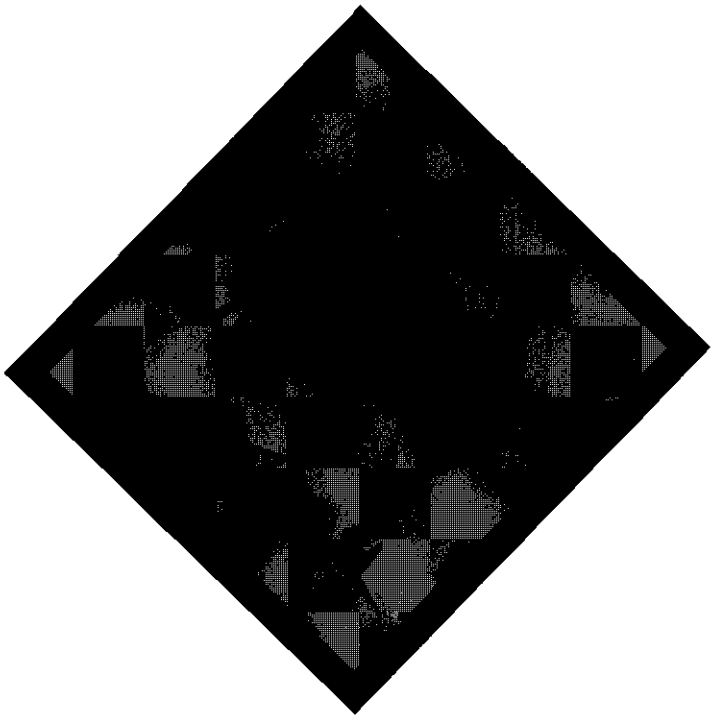

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Abstract

Toxicogenomics is a novel approach integrating the expression analysis of thousands of genes (transcriptomics) or proteins (proteomics) with classical methods in toxicology. Effects at the molecular level are related to pathophysiological changes of the organisms, enabling detailed comparison of mechanisms and early detection and prediction of toxicity. This report addresses the value of the combined use of transcriptomics and proteomics technologies in toxicology. Acute hepatotoxicity was induced in rats by bromobenzene administration resulting in depleted glutathione levels and reduced average body weights, 24 hours after dosage. These physiological symptoms coincided with many changes of hepatic mRNA and protein content. Gene induction confirmed involvement of glutathione-S-transferase isozymes and epoxide hydrolase in bromobenzene metabolism and identified many genes possibly relevant in bromobenzene toxicity. Observed glutathione depletion coincided with induction of the key enzyme in glutathione biosynthesis, γ-glutamylcysteine synthetase. Oxidative stress was apparent from strong upregulation of heme oxygenase, peroxiredoxin 1 and other genes. Bromobenzene-induced protein degradation was suggested from 2-dimensional gelelectrophoresis, upregulated mRNA levels for proteasome subunits and lysosomal cathepsin L, whereas also genes were upregulated with a role in protein synthesis. Both protein and gene expression profiles from treated rats were clearly distinct from controls as shown by principal component analysis, and several proteins found to significantly change upon bromobenzene treatment were identified by mass spectrometry. A modest overlap in results from proteomics and transcriptomics was found. This work indicates that transcriptomics and proteomics technologies are complementary to each other and provide new possibilities in molecular toxicology.

II.1 Introduction

Toxicology aims to detect adverse effects of a compound on an organism based on observed symptoms or toxicity markers. However, a wide variety of mechanisms underlie the different types of toxicity at the cellular level. To discriminate between these various types of toxicity, an extension of the methods and markers currently used in toxicology is required. Technologies based on the progression in functional genomics research enable the determination of the expression of thousands of genes (transcriptomics) or proteins (proteomics) in a single experiment. In the novel research discipline toxicogenomics, functional genomics technologies are integrated with classical methods to study toxicity at the molecular level in relation to the patho-physiological changes of the organism. This holistic approach enables detailed comparison of mechanisms and early detection and prediction of toxicity. Primary effects of toxicants on gene expression are triggered upon binding to receptors, whereas secondary effects on gene expression are to be expected as a result of changes in various cellular processes.

cDNA-microarray based multiple gene expression measurement (transcriptomics) was shown to be a powerful tool in the mechanistic assessment of toxic responses [34];[21];[22];[40];[27]The determination of thousands of gene expression levels simultaneously in a given sample provides an insight in the molecular processes that together determine the specific status of that sample. Gene expression profiles can be used to discriminate samples exposed to different classes of toxicants, to predict toxicity of (yet) unknown compounds and to study cellular mechanisms that lead to or result from toxicity. In analogy to this, the simultaneous measurement of the thousands of proteins in a cell will be of great benefit for toxicology, or even of more importance, while the proteins predominantly act in the cellular reactions rather than the gene transcripts. As all proteins have different properties (mass, iso-electric point, solubility, stability, etc), the accurate measurement of thousands of proteins in a sample is a very complicated task. In this respect, the great advantage of measuring transcripts is that only one type of biomolecule, the mRNA (with all its different sequences), has to be extracted and quantified. Whether all cellular mechanisms can be identified at the mRNA level is still uncertain. For instance, the process of apoptosis is primarily executed through proteolytic (self)activation of the caspase cascade. Especially cell-protective responses might be orchestrated through fast modification or subcellular redistribution of proteins already present in the cell. Proteomics technologies may have a better chance to visualise processes that do not involve active biosynthesis, or at least they will be complementary to gene expression analysis. Although relationships between single gene expression and corresponding protein levels have been widely studied, to this date, not much has been published on the relation between large scale transcriptomics and proteomics assessments. Scarce examples are provided in literature, for instance describing changes in *Bacillus subtilis* [71];[72]. Ruepp et al. [73] describe gene and protein expression in rat liver after acetaminophen (paracetamol) administration, and identified several proteins that changed in acetaminophen hepatotoxicity. It is important to realise that the relationship between mRNA and protein content is heavily dependent on time, cellular localisation as well as stability of the molecules. Transcription of DNA into mRNA is an earlier and more rapid response than the translation from mRNA to proteins. An advantage of the proteomics approach is that it allows the finding of previously unknown molecules, in contrast to the cDNA microarray technology, which only regards known DNA sequences.

This study addresses the value of the functional genomics technologies using cDNA microarrays and 2-dimensional gelelectrophoresis-based proteomics in toxicology. For this evaluation, the well-studied toxicant bromobenzene (BB) was used to induce hepatotoxicity in rats. Bromobenzene is an industrial solvent that elicits toxicity predominantly in the liver, where it causes centrilobular necrosis. Like many xenobiotics, it is a hydrophobic molecule that is subjected to biotransformation in order to enable excretion in the urine. The metabolism and toxicity of bromobenzene in (rat) liver have been described in detail [74,75], [76-79]; Bromobenzene is subjected to cytochrome P450-mediated epoxidation, followed by either conjugation with glutathione, enzymatic hydrolysis or further oxidation of the phenols, leading to hydroquinone-quinone redox cycling. At high BB doses, primarily due to conjugation to the epoxides or bromoquinones, hepatic cellular glutathione is depleted. This triggers a number of secondary reactions like lipid peroxidation, altered intracellular calcium levels, and mitochondrial dysfunction, ultimately leading to cell death. Housekeeping functions such as energy production, (protein) biosynthesis and cytoskeleton organisation might be disrupted. An important process in bromobenzene toxicity is the covalent binding of reactive BB metabolites to (specific) endogenous proteins, especially containing sulfhydryl groups [80]. Necrotic cells elicit responses from neighbouring cells like recruitment or induction of proteases. If the BB-induced damage does not lead to cell death, many genes and proteins involved in recovery and regeneration will be regulated to restore homeostasis. In the liver, the toxic effects are primarily observed at the centrilobular region, due to the high oxygen concentration and cytochrome P450 activity. After transport to the kidneys, bromobenzene induces nephrotoxicity mainly through BB-metabolites conjugated to glutathione that elicit oxidative stress.

A simple study design assesses the molecular toxicity of an intraperitoneal dose of bromobenzene, 24 hours after administration. cDNA Microarray measurements were duplicated and 2-dimensional electrophoresis gels were performed in triplicate for each individual animal, while control groups of both untreated and vehicle treated rats were included. In-depth toxicological discussion of the results is beyond the scope of this evaluation of transcriptomics and proteomics in toxicology, and will be reported elsewhere [3]. Although toxicological interpretations are restricted by the limited design of the studies reported here, a comprehensive set of genes and proteins in rat liver is presented that change upon bromobenzene treatment and that may play a role in the mechanism of toxicity leading to hepatocellular necrosis. Using transcriptomics and proteomics techniques we were able to confirm previously reported effects and identify genes and proteins that were not recognised before in bromobenzene toxicity, involved in processes like drug metabolism, oxidative stress, GSH depletion, the acute phase response and many more yet to be resolved.

II.2 Materials and Methods

Unless otherwise indicated, all reagents were obtained from Sigma. Bromobenzene 99% was from Sigma-Aldrich Chemie GmbH. Corn oil was from obtained from Remia.

In vivo rat study
Male Wistar outbred rats (Charles River Deutschland), (10-12 weeks, body weight 225 ± 8 grams) were injected intraperitoneally with bromobenzene (5.0 mmol/kg body weight, dissolved in corn oil, 40% v/v) or corn oil only, as vehicle control. Rats (3-4 per group) were treated with BB or with corn oil only, and an additional group contained the untreated controls. Rats had free access to food and drinking water and were treated according to Good Laboratory Practice (GLP) guidelines and protocols approved by the laboratory animal ethical committee. Rats were sacrificed by decapitation 24 hours after BB or CO administration. Livers were immediately dissected, quickly weighed, snap-frozen in liquid nitrogen, and stored at –80°C until further processing. Livers were homogenised with a mortar and pestle in liquid nitrogen before preparing the total RNA and total protein extracts. Livers were isolated and processed in a random order to exclude biased results originating from sample preparation.

GSH determination
GSH + GSSG and GSSG levels in liver homogenate were determined using 5,5'dithiobis-2-nitrobenzoic acid (DTNB) to oxidise GSH and form 5-thio-2-nitrobenzoic acid (TNB) which was measured spectrophotometrically at 405 nm. GSSG was measured after derivatisation of GSH with 2-vinyl-pyridine [81,81].

Cytosolic glutathione-S-Transferase activity
Microsomal and cytosolic fractions were isolated by ultracentrifugation from homogenised liver. The activity of glutathione-S-transferase in the cytosol of rat liver was determined using the model substrate CDNB (1-chloro-2,4-dinitrobenzene) according to [82]. Total protein concentration was determined according to the standard Bradford method using Biorad solution [83].

RNA extraction
Total RNA was extracted from liver homogenate using Trizol (Life Technologies S.A) according to the manufacturer's protocol. Total RNA was further purified using the RNEasy RNA purification kit (Qiagen). RNA was checked for purity and stability by gel electrophoresis and the concentration was calculated from the extinction at 260 nm as determined spectrophotometrically.

Reference RNA
To allow comparison of all individual expression patterns, reference RNA was prepared. Dissected organs (liver (~50% w/w of total), kidneys, lungs, brains, thymus, testes, spleen, heart, and muscle tissues) of several untreated male Wistar rats were flash-frozen in liquid nitrogen and stored at –80 °C. All tissues were homogenised together in liquid nitrogen before RNA isolation.

cDNA Microarray preparation
About 3000 different sequence verified rat cDNA clones from the I.M.A.G.E. consortium were purchased (Research Genetics.), and cDNA was amplified by PCR using forward (5' – CTGCAAGGCGATTAAGTTGGGTAAC-3') and reverse (5'-GTGAGCGGATAACAATTTCACACAGGAAACAGC-3') primers containing a 5'- C6-aminolinker (Isogen Bioscience) to facilitate cross-linking to the aldehyde coated glass microscope slides. PCR products were purified by ethanol precipitation and checked for purity and fragment size by electrophoresis on a 1% agarose gel. Purified PCR products were dissolved in 3x SSC and clones were arrayed in a controlled atmosphere on CSS-100 silylated aldehyde glass slides (TeleChem) (modified from DeRisi et al., [48]). In total, about 3800 cDNAs, including 3000 different rat genes, some in duplicate, and various control clones were deposited on the slides. After drying, slides were blocked with borohydrideand stored in a dark and dust-free cabinet until further use. Prior to

hybridisation, slides were prehybridised in buffer (5x SSC, 0.1 % SDS, and Bovine Serum Albumin (10 mg/ml)) for 2 hours at 42°C , washed in milliQ water, dipped in isopropanol and dried.

cDNA synthesis and labelling

A typical labelling reaction was performed using 50 µg of total RNA using an indirect procedure. *In vitro* transcription reactions were performed using reverse transcriptase according to the protocol with the Cyscribe Reagent Kit (Amersham Biosciences). In this reaction, amino allyl dUTP linkers were incorporated, after which the reaction mixture was divided over two portions for coupling with Cy3 or Cy5 fluorophores respectively.RNA was degraded by hydrolysis in NaOH (30 min. at 37°C and the mixture was neutralised with an equimolar amount of acetic acid. The cDNA was purified using the QIAquick spin columns (Qiagen) and water was evaporated. Pelleted cDNA was resuspended in 0.3 M sodium bicarbonate buffer, pH 9.0 and Cy3 or Cy5 fluorophores (freshly dissolved in DMSO) were chemically attached to the aminoallyl linkers. The reaction mixture was quenched with hydroxylamine (0.3 volumes, 4 M) for 1 min. at room temperature to avoid non-specific fluorescence by free fluorophores. Labelled cDNA was purified from unincorporated fluorophores using an AutoseqG-50 (Amersham Biosciences) sephadex chromatography column. The amount of cDNA obtained and the incorporation rate of the fluorophore were determined spectrophotometrically. Prior to hybridisation, labelled cDNAs of both sample and reference were mixed and dissolved in 30 µl EasyHyb hybridisation buffer (Roche Diagnostics). Yeast tRNA (100 µg, Life Technologies S.A) and Poly dAdT (20 µg, Amersham Biosciences) were added to avoid non-specific binding. The hybridisation mixture was denatured for 1.5 minutes at 100 °C and pipetted onto a pre-hybridised microarray slide,covered with a plastic coverslip and, embedded in a slide incubation chamber (Corning, Life Sciences), submerged in a water bath for16 hours at 42°C. After hybridisation, slides were washed by firm shaking in 0.5x SSC buffer in a 50ml tube and two times 10 minutes in 0.2x SSC on a mechanical shaking platform. Slides were dried quickly by centrifugation at 700 rpm.

Scanning

Slides were scanned immediately after drying using the ArrayWorxs CCD scanner (Applied Precision). The ArrayWorxs software processes the acquired images into results files, text files containing signal and local background intensity for both channels. Excel (Microsoft Corporation) was used to further process the data.

Transcriptomics experimental design

In order to compare many samples, individuals, or samples from different studies, an external reference was introduced. Each individual rat sample was co-hybridised with the reference RNA, prepared from untreated rat tissues. For each gene fragment, the amount of mRNA in the sample relative to the amount in the reference was determined. The complete set of hybridisations was duplicated with swapping of the two fluorophores incorporated in the sample and reference RNA. As a consequence of the introduction of the reference sample, comparisons are indirect, as the ratios 'treated / reference' and 'control / reference' are compared to obtain the desired 'treated/control' values. To assess whether different results are obtained using the indirect comparison, three direct co-hybridisations of one BB rat liver and one CO rat liver were performed and compared to the results obtained through indirect comparison of all the BB and CO samples relative to the reference.

Data Analysis

For each spot, the local background intensity was subtracted from the signal intensity. Technical variations introduced during labelling of RNA samples with the two different fluorophores or by scanning have to be taken into account. It is assumed that the large part of the transcripts is equally present in both samples and the total fluorescence should be equal in both channels. Therefore, the sum of all background subtracted signals (if >0) of each channel was normalised to an arbitrary value of 1,000,000 to enable comparison between the channels but also between the microarrays. Background intensities outside the cDNA spots were very low and homogeneous. To account for non-specific hybridisation and background fluorescence of the cDNA on the slide, control spots were included on the microarrays containing cDNAs of fungal or yeast origin, with no significant homology

to (known) rat cDNAs. Fluorescence intensity in these spots was used to determine a signal intensity threshold value. If background-corrected signal intensities were below a threshold value of 100 for any of the two channels, measurements were excluded from analysis. After doing so, one complete microarray, co-hybridised with RNA from BB-treated rat liver and reference RNA, was excluded from further analysis since too many spots did not meet the threshold value. Background-corrected and normalised signal intensities were used to calculate the ratio of gene expression of the sample over the reference. The group average fold change in gene expression relative to reference was calculated for the untreated, corn oil control, and bromobenzene groups (up to 8 values per group). Statistical significance of the difference in expression between these groups was analysed with a two-tailed Student's T-test for each gene, and differences were considered significant if the p-value did not exceed 0.01. The average fold changes of CO versus UT and of BB versus CO were calculated from the group average fold changes.

Proteomics

Sample preparation

Homogenised frozen liver was diluted 1:15 (w/v) with sample lysine buffer consisting of 8M urea, 2% (v/v) CHAPS, 1% (w/v) DTT, 0.8% (v/v) Pharmalyte. Samples werefurther homogenised through a 25 inch gauge needle using a 10 ml syringe. Samples were centrifuged for 30 minutes at 100,000 g at 15°C. Supernatant was stored in aliquotsat –80°C until further use. Protein was determined in homogenised liver using a dye-binding procedure [83,83].

Two dimensional gel electrophoresis, analytical gels

13 µmol of homogenised liver sample (50 µg of protein) was overnight rehydrated in a volume of 350 µmol in a Reswelling Tray on Immobiline DryStrip (IPG) pH 4-7 in 0.5% (v/v) CHAPS, 15 mM DTT, 0.5% (v/v) IPG buffer pH 4-7L. Isoelectric focussing of samples was performed on a Multiphor II Electrophoresis Unit for 45000 VH using the following program: 30 min at 150 V; 1 h at 300 V; 1 h at 1500 V and 12 h and 20 min at 3500 V. Subsequently, IPG strips were equilibrated for 15 min in Equilibration Buffer (6M urea, 30% (w/v) glycerol, 2% SDS in 0.05 M Tris-HCl buffer, pH 8.8) containing 1% (w/v) tributylphospine) and 0.001% (w/v) bromophenol blue. Next, IPG strips were equilibrated for 15 min in Equilibration Buffer containing 250 mM iodoacetamide. Second dimension electrophoresis was performed on custom made 12-15% polyacrylamide gels, using the Hoefer DALT Multiple Casting Chamber (Amersham Biosciences). Gels were prepared according to procedures recommended by the manufacturer. Gels were silver stained without glutaraldehyde, according to the method described by Shevchenko [51]. Scanning of gels was performed on a BioRad GS-710 Calibrated Imaging Densitometer (Bio-Rad).

Gel Image and Data analysis

Scanned TIFF images were analysed using Phoretix 2D Gel Analysis Software version 5.01 (Nonlinear Dynamics). For one of the bromobenzene treated rats, repeated attempts to obtain an analysable gels image were made, but the quality criteria were not met and no proteome data were analysed for this rat. For all other gels, spots were automatically detected and analysed images were scrutinised by eye for undetected or incorrectly detected spots. The total staining intensity within each detected spot, that is the amount of protein present, is defined as 'spot volume'. To compare spot volumes across different gels, the protein spots detected in each experimental gel were matched to the corresponding protein spots within a digitised reference spot pattern. Background intensity was subtracted from the spot volumes. To correct for differences in staining intensity between gels, spot volumes were divided by the volume of all spots and multiplied by the summarised total area of all spots. The resulting values represent the 'normalised spot volumes'.Differences in spot volumes from different treatment groups were analysed for statistical significance by two-tailed Student T-tests (assuming normal distributions and equal variance) using Excel.

Two dimensional gel electrophoresis, preparative gels

Preparative gels for mass spectrometric identification of differentially expressed proteins spots were prepared similar to the analytical gels, except that at least 1 mg of protein was loaded per gel.

Mass spectrometry
For identification, protein spots were manually cut from the gel in a dust free cabinet to prevent contamination with keratin. Gel plugs were processed for mass spectrometry essentially according to Shevchenko [51]. Silver stained gel pieces were destained with 30 mM potassium ferricyanide and 100 mM sodium thiosulphate (Merck), washed with water (MilliQ, Milllipore) and twice with acetonitrile (Biosolved B.V.) The liquid was substituted with reduction buffer (6.5 mM DTT in 50 mM NH_4HCO_3 pH 8.5) followed by a 60minutes incubation at room tempurature. The buffer was then replaced with acetonitrile which in turn was replaced with alkylation buffer (55 mM iodoacetamide in 50mM NH_4HCO_3) and gel plugs were incubated for 30 minutes at room temperature in the dark.The gel pieces were dehydrated with acetonitrile and rehydrated with 50 mM NH_4HCO_3 pH 8.5 twice. Finally the gel pieces were dehydrated with acetonitrile and rehydrated in digestion buffer at 4°C (50 mM NH_4HCO_3,) containing 10ng/µl trypsin, and then incubated overnight at 37 °C. Samples were centrifuged and the supernatant was transferred to micro ZipTip C18 pipette tips (Milllipore), and further processed according to the manufacturer's protocol. For MALDI-TOF-MS, the peptides were eluted directly from the ZipTip onto the MALDI target in 1-2 µl matrix solution (10 mg/ml α-cyano-4-hydroxycinnamic acid in 50% (v/v) acetonitrile, 0.1% trifluoroacetic acid). The peptide mixture for nano-electrospray MS/MS (NANO-ESI-MS/MS) was eluted from the ZipTip in 1-2 µl 50% (v/v) acetonitrile containing 0.1 % (v/v) formic acid and transferred into a custom made nano-electrospray needle. The entire desalted digest from the silver stained spots was used for peptide mapping by MALDI-TOF-MS. When peptide extracts obtained from coomassie blue stained spots were used, the digest was split 1:4 for MALDI-TOF-MS peptide mapping and nano-electrospray MS/MS, respectively. MALDI-TOF-MS peptide mass spectra were recorded on an Applied Biosystems voyager DE-STR mass spectrometerin reflector and delayed extraction mode. The data were sampled with 4 GHz (0.5-ns channel width). All further processing including spectrum calibration based on the trypsin autodigest was performed using the software package DataExplorer 4.0 provided by the manufacturer. For further protein identification, a Micromass Q-tof mass spectrometer with a nanospray source was used for partial sequencing of the ZipTip desalted peptide mixture. A full scan was acquired over a wide mass range and precursor ions of interest were selected for MS/MS ion scan. This technique is well suited to high-sensitivity sequencing because there is no need for further chromatographic separation of the peptide mixture.

Database search mass spectrometry
Proteins were tentatively identified using the Mascot Search software (Matrix Science Ltd) to search the OWL (http://bioinf.man.ac.uk/dbbrowser/OWL/) protein database. The parameters were set to allow 1 miss cleavage with the enzyme trypsin and a peptide mass tolerance of 50-150 ppm. Additional protein information and calculation of theoretical pI and molecular mass were obtained from SwissPROT (http://www.expasy.ch).

Principal component analyses
To discern between transcriptome or protein profiles obtained from rats exposed to bromobenzene and controls, principal component analyses were performed. Principal component analysis (PCA) is a mathematical technique that reduces the many dimensions of a large dataset to a few dimensions that actually describe the large part of the variation in the samples without a priori knowledge. PCA was performed using EAGLES, an in house developed software package. The dataset consisted of all expression ratios of sample versus reference of all genes left after quality filtering. Ratios were log transformed (base 10) for PCA analysis, and excluded values were replaced by a value of 0 . A two-dimensional plot of the most relevant principal components is used to visualise the difference in gene or protein expression profile from the different rat livers, displayed as the objects in the plot. The larger the separation between two objects in the plot, the higher the degree of difference is between the expression profiles.All spot volumes from the three protein gels obtained from each animal were included in the analysis. For each animal, triplicate scores (representing gels) are interconnected by lines resulting in triangles. By comparison of the distances between the centre of each triangles of different animals conclusions can be drawn with respect to the difference in proteomes.

II.3 Results

Rats received a single intraperitoneal dose of bromobenzene and the effect on expression of proteins and genes in the liver was determined using proteomics and transcriptomics technologies. The bromobenzene dose was chosen based on previous studies to be hepatotoxic, and this was confirmed by the finding of a nearly complete glutathione depletion at 24 hours after bromobenzene administration (Figure II.1). Glutathione (GSH + GSSG) levels were lowered 27-fold from nearly 21 µmol/g in the corn oil controls to 0.8 µmol/g in the livers of bromobenzene treated animals. The low level of oxidised (GSSG) relative to reduced glutathione (GSH) indicates that the depletion is primarily due to conjugation and to a much lesser extent due to oxidation of glutathione. No changes in GSH levels were induced by corn oil administration. The bromobenzene administration resulted in on average 7 % decrease in body weight of after 24 hours, whereas vehicle control rats gained on average 6 % of weight. The relative liver weights did not change significantly. A significant average 1.4-fold increase ($p < 0.04$) in activity of glutathione-S-transferase towards CDNB was observed in the cytosolic liver fractions of rats treated with bromobenzene compared to vehicle controls, which showed activities similar to untreated controls.

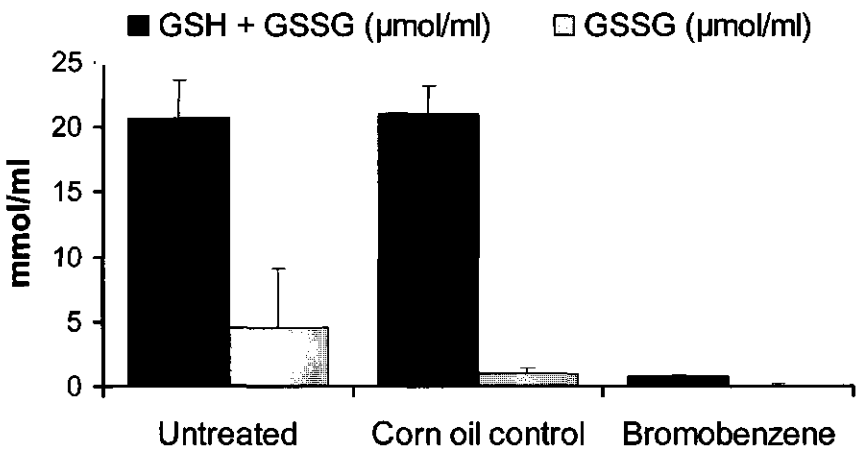

Figure II.1. *Intracellular (GSH + GSSG) and (GSSG) levels*
Intracellular (GSH + GSSG) and (GSSG) levels were determined of rat liver homogenate of untreated, corn oil controls and bromobenzene treated rats. An almost complete depletion of intracellular GSH levels occurred 24 hours after bromobenzene intraperitoneal administration.

Transcriptomics

Using cDNA microarrays, we determined the transcript levels of about 3000 different genes in rat liver, relative to the expression in a reference sample, in duplicated microarray hybridisation experiments for all rats individually. About 10-20% of the rat cDNA spots of each array contained no or very weak fluorescent signal, corresponding to genes that are not expressed or only below detection limits of the technology. After the quality filtering procedure as described in the methods section, the remaining elements were subjected to

extensive analysis. The majority of the genes represented on our microarray was comparably expressed throughout all the samples. However, the bromobenzene treatment distinctly elicited alterations in the expression pattern of a number of genes in rat liver. The average fold changes in expression were calculated between BB treated versus corn oil control (CO) rat livers, and between CO and untreated rat livers. Using the quality criteria described in the Methods section, 32 genes were found to be significantly upregulated, and 17 were repressed more than twofold by bromobenzene treatment relative to the vehicle control, while respectively 63 and 35 genes were up- or downregulated when considering a more subtle (> 1.5) fold change. Genes that changed expression significantly upon bromobenzene treatment were functionally grouped and listed in Table II.1. (drug metabolism, glutathione metabolism and oxidative stress), Table II.2. (acute phase response), Table II.3. (protein synthesis and proteolysis, Table II.4. (others, > 1.5-fold change).

Table II.1. *Genes related to drug metabolism, glutathione metabolism and oxidative stress*

The columns describe the Genbank accession numbers (AC#) of the cDNA fragments present on the microarray, the name of the gene, the average fold change (FC) in expression of both CO versus UT and BB versus CO, as well as the p-value of the Student's T-test.[b]: duplicate cDNA fragment printed on the microarray

		CO / UT	BB / CO	
Acc#	**Gene name**	**FC**	**FC**	**p**
AA900551	Epoxide hydrolase (microsomal) (Ephx1)	0.76	5.59	0.000
AA923966	Aflatoxin B1 aldehyde reductase (Afar)	1.04	4.70	0.000
AA955106	Aldehyde dehydrogenase (Aldh)	1.09	2.20	0.005
AA956507	NADPH-cytochrome P-450 oxidoreductase	0.77	1.78	0.002
AA818917	similar to NADH-ubiquinone dehydrogenase	0.79	1.54	0.010
AA818339	Glutathione S-transferase Ya (Gsta)	0.84	3.70	0.000
AA998734	Glutathione-S-transferase, mu type 2 (Gstm2)	0.97	2.58	0.004
AA965220	gamma-glutamylcysteine synthetase (Gclc)	1.07	2.53	0.006
AA818339 b	Glutathione S-transferase Ya (Gsta)	0.94	2.40	0.006
AA955668	Glutathione S-transferase P (Gstp)	1.00	1.39	0.006
AA819810	Weakly similar to glutathione S-transferase P	1.32	0.46	0.001
AA819810 [b]	Weakly similar to glutathione S-transferase P	1.14	0.38	0.000
AA964788	Glutathione peroxidase (Gpx1)	1.10	0.37	0.001
AA874884	heme oxygenase gene (HO-1)	0.99	5.20	0.001
AA817693	ferritin light chain subunit	0.83	4.29	0.005
AA874884	heme oxygenase (HO-1)	1.06	2.70	0.002
AA875245	Peroxiredoxin 1 (Heme-binding protein 23)	0.96	2.61	0.000
AA875245 b	Peroxiredoxin 1 (Heme-binding protein 23)	0.92	2.04	0.001
AA818441	ferritin-H subunit	0.89	2.01	0.004
AA957593	Tissue inhibitor of metalloproteinase-1 (Timp1)	0.97	1.69	0.004

Table II.1 lists the enzymes involved in drug metabolism that are responding upon BB treatment. Bromobenzene strongly induces the levels of mRNA encoding the liver glutathione-S-transferase Alpha and Mu subunits, the microsomal epoxide hydrolase (Ephx1) and the aflatoxin B1 aldehyde reductase (Afar). Aldehyde dehydrogenase gene expression increased more than twofold upon bromobenzene treatment. Concurrently, the NADPH-cytochrome P-450 oxidoreductase was significantly upregulated. In contrast to the Gst Alpha, Mu and Pi subunits' upregulation, the 'EST weakly similar to (rat) Gst Pi subunit' decreased

over twofold. Sequence analysis of this cDNA fragment (ACC# AA819810) revealed similarity to the rat Gst class Pi 7-7 (gi:121749) at the amino acid sequence level.Also genes involved in the metabolism of intracellular glutathione that changed upon BB treatment are listed in Table II.1. Besides the induction of glutathione-S-transferases, a 2.5 fold upregulation of γ-GCS (Gclc), the key enzyme in GSH biosynthesis was observed. GSH peroxidase, an enzyme also implicated in oxidative stress was found to be downregulated. Genes typically responding to oxidative stress such as heme oxygenase-1, peroxiredoxin 1 (heme binding protein 23), metallothioneins, ferritin, TIMP1 were found to be induced.
Bromobenzene treatment elicits changes in transcript levels of many of the so-called acute phase proteins. Ferritin, apolipoprotein M and metallothionein are induced in the acute phase response (APR) and were found to be strongly induced by the BB treatment. Moreover, negative APPs including alpha-1-inhibitor 3, alpha-1-macroglobulin, transferrin, complement components, liver fatty acid binding protein 1 and fibrinogen are downregulated 24 hours after bromobenzene treatment (Table II.2.)
Interestingly, many ribosomal subunits and other factors involved in protein synthesis (Initiation factor, elongation factor) were induced upon BB treatment (Table II.3). Furthermore, table II.3 lists the induction of several components of the proteasome and of the proteolytic enzyme Cathepsin L from the lysosome. Many genes with interesting implications in other cellular processes (Table II.4) were found to be upregulated such as beta actin, glucose-6-phosphate dehydrogenase, thyrotropin receptor, cytochrome c, cytochrome b5, cysteine sulfinic acid decarboxylase and clathrin. Genes downregulated by BB include a mitochondrial ATPase inhibitor, thymosin beta 10, betaine homocysteine methyl transferase (Bhmt), cysteine dioxygenase, hemoglobin, insulin-like growth factor and mitochondrial HMG-CoA synthase.

Table II.2. *Genes related to the hepatic acute phase response*
The columns describe the Genbank accession numbers (AC#) of the cDNA fragments present on the microarray, the name of the gene, the average fold change (FC) in expression of both CO versus UT and BB versus CO, as well as the p-value of the Student's T-test.[b]: duplicate cDNA fragment printed on the microarray

		CO / UT	BB / CO	
Acc#	**Gene name**	**FC**	**FC**	**p**
	ferritin light chain subunit,	0.83	4.29	0.005
AA874884	heme oxygenase (HO-1)	1.06	2.70	0.002
AA900218	metallothionein-i (mt-1)	1.46**	2.76	0.002
AA859399	Metallothionein	1.86**	2.71	0.002
AA858741	apolipoprotein M	1.21	2.05	0.001
AA818441	ferritin-H subunit	0.89	2.01	0.004
AA819204	Weakly similar to rat Metallothionein-I	1.14	1.66	0.007
AA926277	CELF/ C/EBP delta transcription factor	0.93	0.83	0.007
AA817963	alpha-1-inhibitor 3	0.62**	0.64	0.008
AI072074	Fatty acid binding protein 1, liver	0.85	0.56	0.001
AA817963 [b]	alpha-1-inhibitor 3	0.66	0.55	0.005
AA998796	Complement component C9 precursor	1.21	0.54	0.001
AA956238	Vitronectin	1.29	0.54	0.002
AA900592	Similar to human complement component	1.23	0.53	0.000
AA901379	*Inter-alpha-inhibitor H4P heavy chain*	1.52*	0.52	0.009
AA819267	Fibrinogen gamma chain-a	0.98	0.52	0.007
AI044677	Weakly similar to rat gamma-fibrinogen	1.84**	0.51	0.004
AA963164	Synaptojanin II	1.00	0.49	0.004
AA819043	Fibronectin 1	0.88	0.47	0.000

AI137907	Alpha-1-macroglobulin	1.22	0.46	0.006
AA858975	Transferrin	0.92	0.46	0.006
AA819465	apolipoprotein C-III	0.96	0.44	0.000
AI072074	Fatty acid binding protein 1, liver	1.09	0.42	0.006
AA963445	selenoprotein P	1.10	0.39	0.002

Table II.3 *Genes related to protein synthesis and proteolysis*
The columns describe the Genbank accession numbers (AC#) of the cDNA fragments present on the microarray, the name of the gene, the average fold change (FC) in expression of both CO versus UT and BB versus CO, as well as the p-value of the Student's T-test.[b]: duplicate cDNA fragment printed on the microarray

		CO / UT	BB / CO	
Acc#	**Gene name**	**FC**	**FC**	**p**
AI045792	Highly similar to rat 60S ribosomal protein L9	1.14	2.47	0.010
AA819544	ribosomal protein S2	1.04	2.33	0.001
AA900657	60S ribosomal protein L7A	0.96	2.25	0.000
AA874997	ribosomal protein S8	1.04	1.97	0.001
AA924912	ribosomal protein L6	0.86	1.94	0.000
AA899448	ribosomal protein S3a	0.78	1.89	0.006
AA818640	Highly similar to human 40S ribosomal prot S18	0.88	1.88	0.006
AA925200	60S ribosomal subunit protein L35	0.93	1.85	0.007
AA866268	Highly similar to RL860S ribosomal protein L8	1.10	1.71	0.002
AA818442	Ribosomal protein S11	0.89	1.70	0.001
AA900527	ribosomal protein S26	0.92	1.52	0.009
AA924882	ribosomal protein L15	0.98	1.41	0.005
AA998895	Nucleophosmin (nucleolar protein B23)	0.95	3.02	0.000
AA819244	Nuclear-encoded mitochondrial elongation factor G	0.51	2.31	0.004
AA874949	Similar to human initiation factor eIF-5A gene	0.88	1.95	0.001
AA875102	Highly sim. to mouse/human small nuclear ribonucleoprotein E	0.85	1.62	0.001
AA964651	Highly similar to mouse prefoldin subunit 2	0.77	1.60	0.010
AA899472	Highly similar to eukaryotic translation initiation factor 3. Subunit 8	0.89	1.58	0.000
AA859498	cathepsin L	0.83	3.53	0.037
NA	cathepsin L	0.94	3.21	0.007
AA859484	Proteasome subunit beta type. 3	0.96	2.27	0.000
AA859300	Proteasome subunit alpha type 1	1.00	1.73	0.014
AI030596	Similar to human 26S proteasome subunit S5B	1.15	1.60	0.054
AA859582	Proteasome subunit beta type 5 (epsilon chain)	1.30	1.57	0.057
AI043800	ESTs, Highly similar to 26S proteasome-associated pad1 homolog	0.86	1.47	0.043
AA964414	proteasome component C8	0.92	1.40	0.089
AA925795	proteasomal ATPase (SUG1)	0.96	1.30	0.054

Table II.4 *Genes related to other functions*
The columns describe the Genbank accession numbers (AC#) of the cDNA fragments present on the microarray, the name of the gene, the average fold change (FC) in expression of both CO versus UT and BB versus CO, as well as the p-value of the Student's T-test.[b]: duplicate cDNA fragment printed on the microarray

		CO/UT	BB/CO	
Acc#	**Gene name**	FC	FC	P-value
AI059976	Vesl-2	0.94	3.77	0.001
AA964496	Highly similar to actin beta	1.14	3.48	0.005
AA858998	RAN. member RAS oncogene family	0.91	2.88	0.004
AI030685	nestin	0.98	2.44	0.007
AI060085	Thyrotropin receptor	1.10	2.36	0.002
AA964725	Highly similar to mouse Flightless I homolog	1.18	2.35	0.008
AA965078	mitochondrial 3-2 trans-enoyl-CoA isomerase	1.01	2.22	0.006
AI045953	Cysteine sulfinic acid decarboxylase	1.02	2.17	0.004
AI059951	Neuronal cell death related gene in neuron -7	1.10	2.01	0.001
AA818887	MHC class I	0.92	1.96	0.004
AA956323	membrane bound cytochrome b5	0.78	1.90	0.000
AI058620	Bax inhibitor-1 (BI-1) (Testis enhanced gene transcript)	1.09	1.89	0.002
AI058566	Peptide/histidine transporter	1.06	1.82	0.001
AA874955	Clathrin light chain (LCB3)	0.78	1.80	0.001
AA900595	Anti-silencing protein ASF1 homolog	1.10	1.80	0.000
AA964548	Highly similar to rat tubulin beta chain	0.99	1.79	0.000
AA866442	Somatic cytochrome c	0.64	1.77	0.006
AA900102	Mitochondrial import inner membrane translocase	1.00	1.74	0.005
AA899102	Glucose-6-phosphate dehydrogenase	0.98	1.73	0.003
AA874837	Phosphoglycerate mutase B isozyme PGAM	0.82	1.73	0.007
AA819314	Initiation factor 2 associated 67 kDa protein; methionine aminopeptidase	0.78	1.72	0.010
AA859476	Cofilin	0.81	1.69	0.006
AI453996	Heat shock 70kD protein 5	0.96	1.68	0.004
AA964657	EST. unknown	0.96	1.64	0.005
AA964651	Highly similar to mouse prefoldin subunit 2	0.77	1.60	0.010
AA875070	alpha-prothymosin	0.94	1.58	0.006
AI043642	potassium channel (KCNQ2)	1.00	1.53	0.003
AA957012	Highly similar to human RNA polymerase II	1.00	0.68	0.001
AA866264	similar 20-alpha-hydroxysteroid dehydrogenase	0.92	0.66	0.010
AI070597	Highly similar to human CGI-97 protein	1.60**	0.65	0.009
AA925346	Cytochrome P450 51, lanosterol 14-alpha-demethylase	1.10	0.61	0.000
AA818579	cysteine dioxygenase	0.76	0.58	0.009
AA963258	insulin-like growth factor I (IGF-I)	1.12	0.55	0.009
AA819034	alpha interferon inducible protein	1.31	0.54	0.008
AI136048	mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase	1.45	0.53	0.008
AA819784	Hemoglobin. alpha 1	1.39	0.52	0.010
AA926010	long-chain acyl-CoA synthetase	1.12	0.46	0.002
AA901407	betaine homocysteine methyltransferase (BHMT)	0.67	0.45	0.003
AA924288	thymosin beta-10 gene	0.77	0.42	0.005
AA858662	tryptophan 5-monooxygenase activation protein (Ywhaz)	0.82	0.41	0.000
AA819164	Mitochondrial IF1 ATPase inhibitor	0.96	0.41	0.005
AA926359	receptor-linked protein tyrosine phosphatase	1.18	0.39	0.000

Table II.5. *Genes that change significantly ($p \leq 0.01$) changed more than 1.5 fold by corn oil administration as compared to untreated.*

		CO / UT		BB / CO	
Acc#	**Gene name**	**FC**	**p**	**FC**	**p**
AA964554	Highly similar to human U3 snoRNP-associated 55-kDa protein	2.54	0.009	0.59	0.164
AA859399	Metallothionein	1.86	0.008	2.71	0.002
AI044677	Weakly similar to rat gamma-fibrinogen	1.84	0.010	0.51	0.004
AI070597	Highly similar to human CGI-97	1.60	0.002	0.65	0.009
AA900218	Metallothionein-i (mt-1)	1.46	0.006	2.76	0.002
AA957923	mast cell protease II gene	0.68	0.001	1.25	0.108
AA818896	Cytochrome P-450j (Cyp2e1)	0.63	0.010	1.39	0.128
AA817963	alpha-1-inhibitor 3	0.62	0.007	0.64	0.008

Only a few liver transcripts markedly changed in expression caused by corn oil injection. (Table II.8.) Metallothionein, the "EST weakly similar to (rat) fibrinogen gamma", and C4 complement protein were significantly upregulated by CO. These are proteins modulated in the acute phase response and interestingly, the latter two were downregulated by BB. (Table II.4.) The negative acute phase protein alpha-1-inhibitor was significantly downregulated in the CO controls, and levels decreased further in the BB treated rats. The mRNA for cytochrome P450 oxygenase 2E1 (Cyp2e1) was downregulated on average 1.6 fold by corn oil.

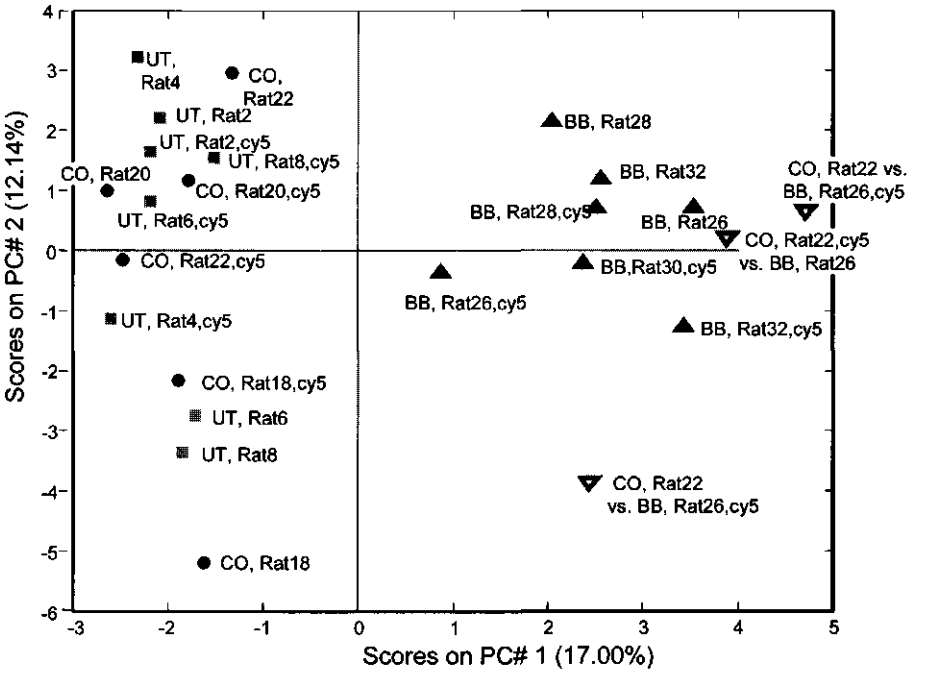

Figure II.2. *Principal component analysis of transcriptome data.*
Objects in the plot represent expression profiles from individual rat livers. The two principal components explaining the majority of the variation in the dataset are plotted. Clearly, the expression profiles of rat liver after BB treatment are distinct from the controls. The injection of corn oil only as the vehicle control did not result in aberrant gene expression profiles compared to the untreated samples. UT = untreated (squares); CO = corn oil vehicle control (circles); BB = bromobenzene treated (triangles) ; Directly compared BB vs. CO samples (inverted triangles) Individual rat numbers are indicated, followed by 'Cy5'when the liver RNA sample was labeled with Cy5 fluorophore (and the reference with Cy3). In the other samples, the fluorophore incorporation was swapped.

Principal component analysis (PCA) was applied to visualise the differences in the expression profiles from treated and non-treated rats (Figure II.2.). The expression profiles of rat liver after BB treatment are clearly distinct from the controls. In the plot, the horizontal distance (principal component # 1) between the groups of BB treated and control liver expression profiles is larger than the distance between samples within one group. The injection of corn oil only as the vehicle control did not result in aberrant gene expression profiles compared to the untreated samples. The vertical distance (PC#2) between samples in the plot does not reflect relevant biological effects but technical variation. The distance between two expression profiles obtained from the same rat indicates that the technical variation introduced by the RNA labelling and hybridisation procedures provides the main source of variation within a treatment group. The interindividual biological variation within the treatment groups did not exceed the technical variation. Additionally, the principal component

values were calculated per gene and genes were ranked according to the PC#1 values. The genes with the largest PC#1 values are differentially expressed after treatment. Genes with positive PC#1 values proved to be upregulated by bromobenzene, whereas genes with negative values were downregulated by BB compared to controls. The genes that account for most of the differences in the expression profiles of BB-treated compared to controls in the PC-analysis were found to be the identical genes that were identified by fold-change calculations (Tables II.1– II.4).

PC-analysis indicated a cluster of genes which most pronouncedly contributed to bromobenzene induced hepatotoxicity. This cluster contained several drug metabolising enzymes like the microsomal epoxide hydrolase 1 (Ephx1), glutathione-S-transferase Alpha (Gsta), Mu (Gstm) and Pi subunits and aflatoxin B1 aldehyde reductase (Afar). Moreover, proteins related to oxidative stress response like ferritin (both heavy and light subunit), heme oxygenase-1 (HO-1), peroxiredoxin 1 and NADPH cytochrome P450 oxidoreductase were elevated in concordance. Most of the genes considerably downregulated were identified as acute phase proteins.

To test whether similar results are obtained using direct or indirect hybridisations including a general reference sample, three co-hybridisations of one BB rat liver and one CO rat liver mRNA sample were performed and compared to the results obtained through indirect comparison of all the BB and CO samples. Relative expression levels should correlate well for all genes, but especially the differential expression of genes should be identified similarly using both approaches. Therefore, the BB/CO fold change of 165 genes identified in the indirect approach to be differentially expressed with > 1.5 fold change (T-test $p < 0.02$) was compared to the average fold change as measured in three replicated BB vs. CO co-hybridisations, resulting in a correlation coefficient of 0.879.

Proteomics

At least three 2-dimensional gels were prepared from each liver extract. A reference protein pattern was constructed from rat liver, containing 1124 protein spots. In Figure II.3, the protein pattern obtained from an untreated animal is shown. Spot numbers and identities - whenever available - of 24 proteins that were differentially expressed in bromobenzene or corn oil treated animals are indicated.

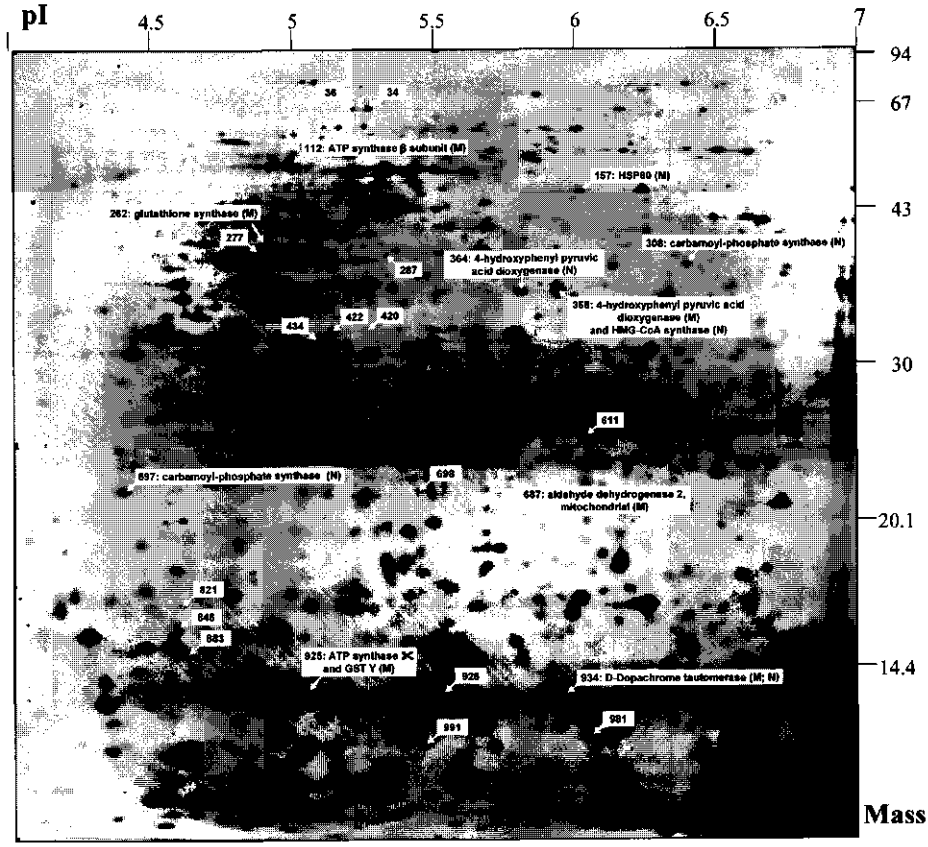

Figure II.3. *Two-dimensional electrophoresis gel of liver from untreated rat*
A two-dimensional electrophoresis gel obtained from an untreated animal is shown. Spot numbers and identities - whenever available - of 24 proteins that were differentially expressed in bromobenzene-treated animals are indicated. The method of identification is indicated: M: MALDI-TOF-MS; N: ESI-MS/MS.

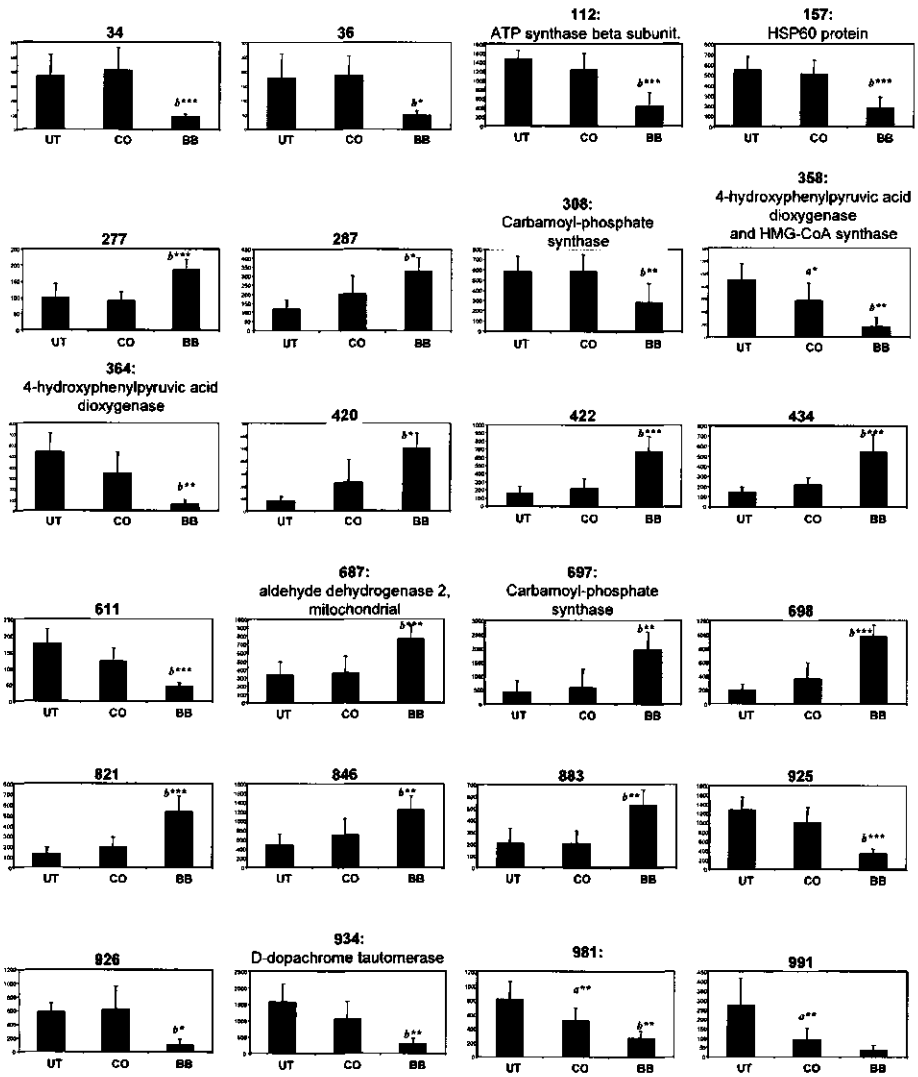

Figure II.4. *Protein spots that changed statistically significant upon corn oil (CO), or bromobenzene (BB) treatment.*
Average spot volume ± standard deviation obtained from all gels within a group is shown. (UT: untreated; CO: corn oil vehicle control; BB: bromobenzene). a: significant difference (two-tailed Student's T-test) between untreated and corn oil vehicle control treated animals. b: significant difference (two-tailed Student's T-test) between bromobenzene and corn oil treated animals. *: $P < 0.05$; **: $P < 0.005$; ***: $P < 0.001$. Protein names of identified spots are indicated.

Figure II.4 displays the volumes for all spots identified as statistically significantly changed upon bromobenzene or corn oil treatment. The bars represent normalised spot volumes, averaged for all (9-12) gels within one treatment group (untreated, vehicle control or bromobenzene treated). Hence, the depicted standard deviation reflects both the interanimal variation, as well as technical variation between gels. Part of the spots marked as significantly changing were identified by MALDI-TOF or nano-electrospray mass spectrometry. Spot 112 was identified as the ATP synthase beta subunit (gi:1374715). The level of ATP synthase beta subunit decreased ~3.5 fold upon bromobenzene treatment. Peptide fragments from spot 925, which also decreased by bromobenzene were found to match the mitochondrial ATP synthase alpha subunit (liver isoform) (gi:114523). Remarkably, other peptide fragments isolated from this same spot matched the glutathione S-transferase Y subunit (gi:204503). The peptide fragment pattern obtained from protein spot 157 was found to match a protein sequence from the murine Heath Shock Protein 60 (gi:51452). Sequence alignment (BLAST at NCBI) indicated that the amino acid sequence is more than 99% identical to the Rattus norvegicus Hsp60 (gi:1334284). The expression of Hsp60 decreased about 3-fold upon bromobenzene treatment as compared to control groups. Peptide fragments from both spot 358 and spot 364 were identified as 4-hydroxyphenylpyruvic acid dioxygenase (Hpd, gi:8393557), using MALDI-TOF-MS and ESI-MS-MS respectively. Hpd converts 4-hydroxyphenylpyruvate dioxygenase to homogentisate and is involved in tyrosine catabolism. Hpd decreased ~5.5 fold upon treatment with bromobenzene, part of which could be explained by an effect of corn oil. Both spots represent proteins with similar molecular mass, however, a slight difference in pI was found. Additional peptide fragment analyses, using ESI-MS-MS, of spot 358 isolated from two new gels, twice revealed a mixture of fragments that matched Hpd and peptide fragments that matched hydroxymethylglutaryl-CoA synthase (HMG-CoA synthase, gi:123330). Spot 687 was identified as mitochondrial aldehyde dehydrogenase 2 (gi:14192933). Aldehyde dehydrogenase 2 increased ~2.5 fold upon bromobenzene exposure, whereas intraperitoneal administration of corn oil did not induce changes in the protein content. Peptide fragments derived from spot 934 matched with fragments from the D-dopachrome tautomerase gene (Dopa, gi:13162287). Dopa is an enzyme catalysing the tautomerisation of D-dopachrome to 5,6-dihydroxyindole. D-Dopachrome tautomerase decreased upon corn oil treatment, but not statistically significant. However, a significant further decrease in expression level was caused by bromobenzene treatment. Spots 308 and 697 both were identified as carbamoyl-phosphate synthase (CPSI, gi:117492), an enzyme involved in the urea cycle (E.C. 6.3.4.16). Spot 308, corresponding with a high molecular mass protein (~40 kDa), decreases upon BB treatment, whereas spot 697 corresponds with a smaller (~25 kDa) protein which amount increases upon BB. One bright spot, 262, was found to be induced by BB 1.5 fold compared to corn oil and 2-fold compared to UT, but no statistical significance could be shown due to one outlier intensity value in the CO group, resulting in standard deviation of over 50% for the CO group. Mass spectrometry identified peptide fragments of this spot matching to glutathione synthetase (gi:1170038), an enzyme involved in the GSH synthesis. Besides the few proteins found to statistically significantly change with corn oil and bromobenzene treatment, a decrease in the average size of the proteins in the bromobenzene livers as compared to the corn oil livers is observed in figure II.5.

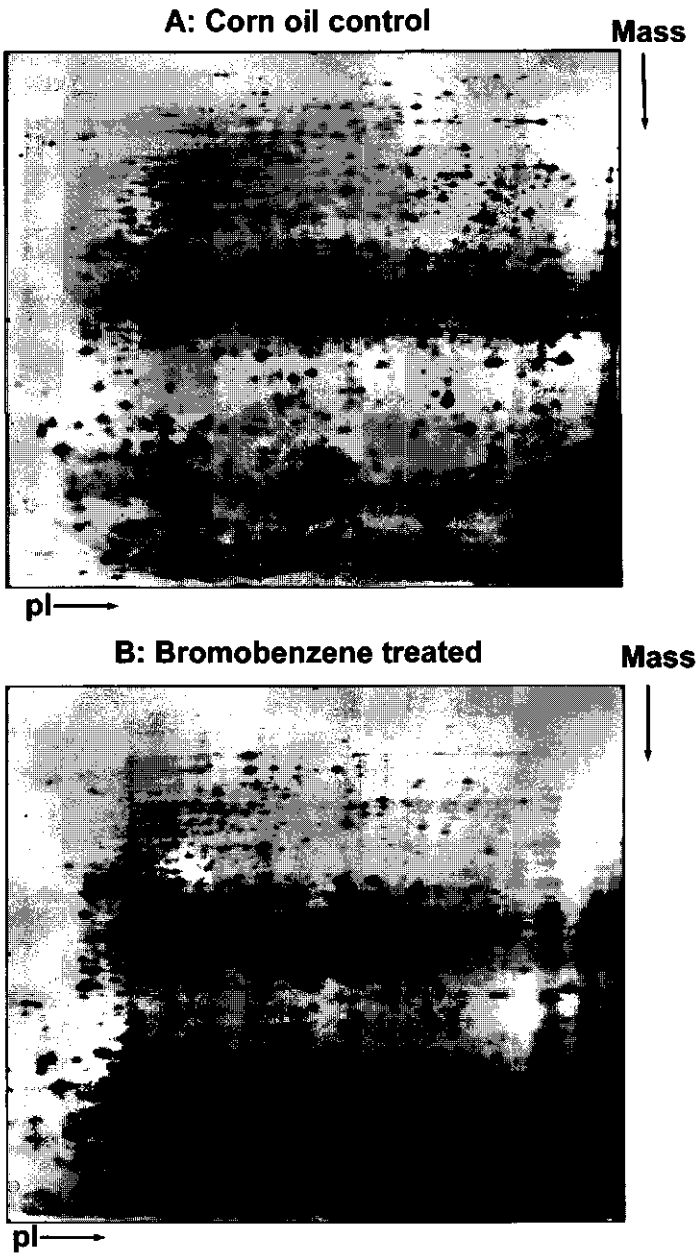

Figure II.5. *Representative gel images from livers of control (A) and bromobenzene-treated rats (B).*
These images show a shift towards proteins with lower molecular mass after bromobenzene treatment.

In all gels from BB-treated livers, less spots are present in the upper (large mass proteins) region of the gels and more spots appear in the lower region compared to both CO and UT controls. Principal component analysis on the proteomics data (Figure II.6) also illustrates a clear difference between liver protein patterns of bromobenzene-treated animals and controls. Each object in the plot represents all normalised spot volumes from one gel. There is a relatively large distance between objects of the BB treatment group to the objects of both control groups. The distance between the three objects from the same rat is generally larger than distance between samples from different animals, indicating that technical variations exceed the interindividual variations within treatments groups.

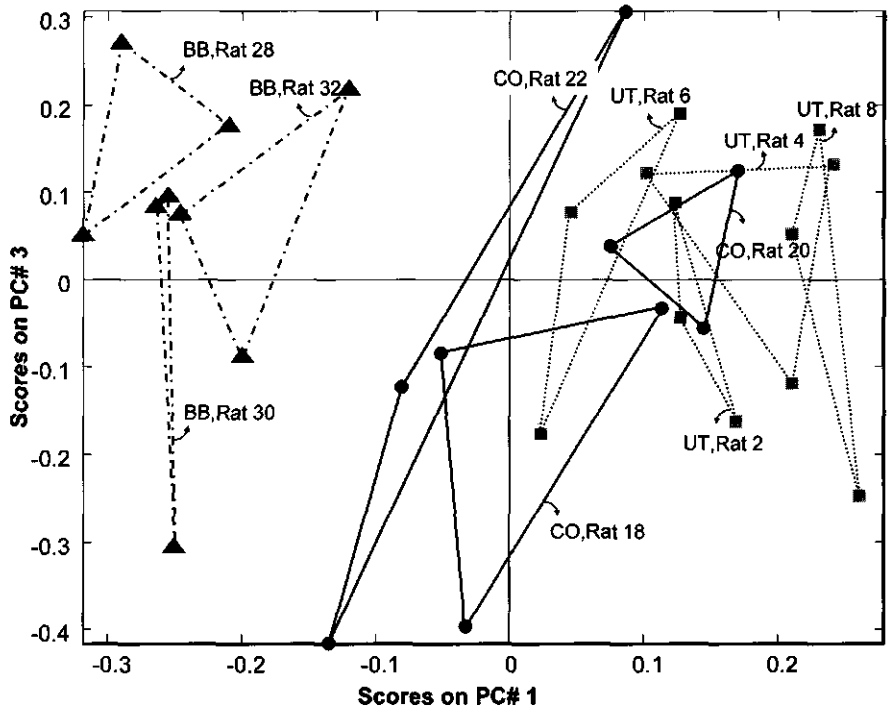

Figure II.6. *Principal component analysis of proteomes.*
PCA was applied using spot volume data obtained from untreated, corn oil control and bromobenzene-treated animals. The two principal components explaining the majority of the variation in the dataset are plotted. Individual gels of bromobenzene treatment samples are displayed by triangles, circles indicate the corn oil controls and squares the untreated samples. The three gels from the same animal are connected by lines. Protein patterns from bromobenzene treated animals are clearly distinct from corn oil and untreated controls. A slight difference in the pattern of corn oil treated animals as compared to untreated rats could be concluded from this image.

II.4 Discussion

Novel functional genomics technologies are applied to toxicology to aid the study of cellular mechanisms of toxicity. The combined application of transcriptomics and proteomics methods was evaluated in a toxicological experiment in rats with a typical hepatotoxicant, bromobenzene. Acute toxicity by intraperitoneal bromobenzene administration is known to lead to centrilobular necrosis in the liver. In elaborate studies of the effects on rat liver gene expression with multiple doses of bromobenzene and time points of analysis, we determined expression profiles that correspond well with the results presented here; a comparable oral dose (5 mmol / kg BW) of bromobenzene elicited distinct responses at 24 hours, while 6 hours after dosage, responses were not yet pronounced. Administration of lower doses of bromobenzene induced fewer changes in the transcriptome. Changes at the expression level are related to other toxicity parameters [3].

The observed glutathione depletion and body weight decrease 24 hours after dosage are physiological symptoms that concur with many changes at the mRNA and protein level in the liver, presented in this report. The depletion of cellular GSH content can not be explained by the formation of GSSG from oxidation of two GSH molecules, since the GSSG content does not increase. Rather, GSH depletion reflects the exhaustive use of GSH in conjugation to reactive BB metabolites catalysed by Gsts, or through spontaneously conjugation. The strong induction of Gst Alpha, Mu and Pi subunit mRNAs that was found (Table II.1) corroborates the importance of GSH conjugation by these enzymes in the biotransformation of bromobenzene. The significant 1.4-fold increase in cytosolic Gst activity towards CDNB, 24 hours after BB dosage, confirms the functional implications of the observed induction of Gst isozymes at the mRNA level. Catalysis of CDNB turnover is effected by several cytosolic Gst isozymes, while Gst Alpha is the most abundant enzyme in rat liver and presumably responsible for the large part of activity towards CDNB. The pivotal role of the depletion of intracellular GSH levels in the process of bromobenzene toxicity was strengthened by the finding of the induced gene expression of γ-glutamylcysteine synthetase, which is the rate-limiting enzyme in GSH synthesis. Induction has been reported under conditions where cellular GSH was depleted, and provides the cell with the ability to reconstitute GSH levels. The other enzyme important in the GSH synthesis, GSH synthase, was not found to change significantly at the mRNA level. However, at the protein level, a bromobenzene-induced increase in GSH synthase (spot 262) was found although one outlier value in the control prevented statistical corroboration of this change. Additionally, the downregulation of GSH peroxidase is probably directly related to GSH depletion and has been shown in acetaminophen toxicity as well [84]. Thus, both transcriptomics and proteomics results provide complementary information to the changes in biochemically determined cellular GSH levels and specific enzyme activity of Gst in the cytosol.
The induction of mRNA for microsomal epoxide hydrolase is coherent with its role in bromobenzene metabolism; the hydrolysis of the toxic epoxide BB intermediates. Microsomal epoxide hydrolase is expressed strongly in liver, but also foundin many other organs. It is induced by xenobiotics like phenobarbital, trans-stilbene oxide and Aroclor 1254 [85]. The cytosolic epoxide hydrolase gene expression was not significantly changed upon bromobenzene treatment. The co-ordinate response of a cluster of genes upregulated by BB consisting of genes like Gsta, Gstm, γ-GCS (Gclc), HO-1 and ferritin, peroxiredoxin1 and NADPH-cytochrome P450 oxidoreductase might reflect a common regulatory mechanism.

Indeed, many of the genes in this cluster have been shown to be under transcriptional control of the Electrophile Response Element (EpRE, formerly named antioxidant response element, ARE). [86,87]. We speculate that more genes in this cluster could be under transcriptional control of the EpRE, probably activated by the BB epoxide metabolites, which are very strong electrophilic species. Heme oxygenase upregulation is a characteristic response to hepatic oxidative stress. Hypoxia, endotoxins, and xenobiotics that induce oxidative stress (e.g. cadmium chloride [88], acetaminophen[84] were all found to induce heme oxygenase. The upregulation of hepatic heme oxygenase 1 by bromobenzene has been described long ago [89]. HO-1 is the enzyme that catalyses the degradation of heme into biliverdin, and induction could be functional against oxidative stress [90]. Bilirubin, produced from biliverdin by biliverdin reductase is an antioxidant, which would provide the cell with a defence mechanism against reactive oxygen species. The co-induction of HO-1 and peroxiredoxin1 was described before after exposure to heme or heavy metals CdCl2 and CoCl2 [91].

The intraperitoneal administration of a high dose of bromobenzene clearly elicits a response well-known as the Acute Phase Response (APR), as can be concluded from the differential expression of the genes listed in Table II.4. The APR is a response of the organism to various types of stress like mechanical damage (liver regeneration after partial hepatectomy), systemic inflammation (endotoxins like lipopolysaccharides) or local inflammation [92,93]. Especially in the liver, the APR coincides with dramatic changes in a wide variety of proteins with the general purpose to provide a protective response and re-establish cellular homeostasis. A major function of the liver, the production of secreted proteins like plasma proteins, is heavily disturbed. The so-called negative acute phase proteins are downregulated in the APR, and comprise plasma transport proteins like albumin, hemopexin, ceruloplasmin and alpha1-acid glycoprotein. The expression of genes that encode for proteins with protective effects, for instance sequestering of reactive oxygen species, is upregulated in APR. Proteins like Ferritin, metallothioneins, and alpha-fetoprotein are regarded as positive acute phase proteins. The injection of corn oil as a control elicits the downregulation of the alpha-1-inhibitor and the upregulation of one metallothionein species. Remarkably, fibrinogen is upregulated by corn oil, in contrast to the downregulation by BB.

The expression of 260 genes in liver and kidney of mice exposed to bromobenzene has been measured and compared to treatment with various chemicals by Bartosiewicz *et al.* [94]. A very high dose of BB (2.5 g / kg BW) was given, causing all mice to die within 48 hours post-dosage. Indeed, the approximate lethal dose in mice was reported to be 0.9 g / kg [95].The authors compared BB toxicity to CCl_4-induced toxicity and reported 15 genes to be regulated 24 hours after administration, both in BB as well as in CCl_4-treated mice, including strong upregulation of hsp25, c-jun, gadd45, γ-GCS and downregulation of Cyp2e. Also, some genes were found to be regulated by BB but not by CCl_4, including Gst Yc, Cyp2b9, myeloid differentiation and P450 reductase. Several genes were identified to change in concordance with our findings, (eg. γ-GCS and glutathione-S-transferases), however, patterns of gene expression also showed marked differences. Bartosiewicz *et al.* found more genes related to general stress pathways to be induced, possibly because a very high dose of bromobenzene was given. Moreover, mice were used whereas rats were studied in our work, which could account for species-specific differences in response.
A correlation was found for the gene and protein expression changes in aldehyde dehydrogenase. At the protein level, bromobenzene induced a 2.5-fold increase in the

mitochondrial aldehyde dehydrogenase 2. Using cDNA microarrays, significant (over twofold) changes in the expression level of aldehyde dehydrogenase and also of aflatoxin B1 aldehyde reductase were found. The induction of aldehyde dehydrogenase and reductase may be an adaptation both at the gene and protein level to increased levels of 4-hydroxy-2-nonenal that could result from bromobenzene induced lipid peroxidation [96];[97].
The 3.5 fold decrease in ATPase beta subunit protein concentration upon bromobenzene treatment probably indicates mitochondrial toxicity and a loss of cellular energy production in line with mitochondrial damage. Previous studies indicated the ability of bromobenzene to deplete ATP-levels *in vitro* and *in vivo* [98];[99]. The protein decrease could not be related to changes in mRNA levels, as the cDNA microarray measurements for the ATPasesubunits were inconclusive. Along with the ATPase decrease, the gene expression of the mitochondrial IF1 ATPase inhibitor was strongly inhibited. Recently, it was shown that acetaminophen-induced hepatotoxicity also resulted in decreased ATP-synthase subunit and Hsp60 levels in mice [100].
Upon BB treatment, the amount of Heat shock protein 60 (Hsp60), 4-hydroxyphenylpyruvic acid dioxygenase (Hpd) and D-dopachrome tautomerase (Dopa) decreased. Remarkably, two spots represent Hpd protein with similar molecular mass but different pI, suggesting that Hpd exists in more than one state, possibly through post-translational protein modification. ESI-MS-MS analyses of spot 358 cut from two new gels revealed both fragments that matched Hpd as well as peptide fragments that matched hydroxymethylglutaryl-CoA synthase. The decrease in intensity of this spot can not be directly related to decreased expression of either Hpd or HMG-CoA synthase as both might contribute to the decrease. Downregulation of Hpd was concluded from analysis of protein spot 364, whereas 1.9 fold downregulated gene expression was found for HMG-CoA synthase. Dopa is catalysing the tautomerisation of D-dopachrome to 5,6-dihydroxyindole, which is a melanin precursor. Dopa is related to macrophage migration inhibitory factor (MIF) both by sequence and by enzyme activity. Moreover, MIF acts as a phenylpyruvate tautomerase, and is expressed at sites of inflammation, with a suggested role in regulating macrophages in host defence. Whether the change in Dopa levels has any toxicological implications related to MIF functions is unclear. A putative toxicological explanation for the observed decrease in Hsp60 and Hpd has yet to be determined. The decrease in the carbamoyl-phosphate synthase (CPSI) of ~40 kDa and the concordant increase in the smaller (~25 kDa) protein upon BB treatment propose a degrading activity towards this protein and provide an example of the capability to visualise protein processing with 2-dimensional gelelectrophoresis. No statistically significant changes were observed in the gene expression levels of Dopa and Hpd (clones AA924020 and AA900788, respectively, on the cDNA microarray) upon bromobenzene treatment. Unfortunately, cDNAs representing Hsp60 and CPSI were not present on the microarray.

Proteomics data indicated that bromobenzene induced a shift in the protein pattern from high molecular mass proteins towards more lower molecular mass species (Figure II.5), which could indicate bromobenzene-induced degradation of proteins. All samples were processed randomly and the shift was only observed in the treated samples. The mRNA level for the lysosomal proteolytic enzyme cathepsin L significantly increased 3.5 fold upon BB injection, and many subunits of the proteasome complex were also found to be induced, although statistical probabilities were lower (Table II.6). The bromobenzene-specific degradation of proteins through induced proteasome and cathepsin L activity might provide an explanation for the protein pattern shift observed in the gels. Besides, bromobenzene metabolites are

known to covalently interact with protein sulfhydryl groups [80]. This protein adduction may target proteins for ubiquitinylation, resulting in activation of an ATP/ubiquitin-dependent proteolytic pathway mediated by the proteasome complex. Induced transcription of many factors involved in protein synthesis such as ribosomal proteins, translation initiation and elongation factors, could be an indication of increased protein synthesis (Table II.5). Together with increased degradation, a higher protein turnover rate is suggested. Alternatively, the mechanism of protein synthesis could be affected in bromobenzene toxicity resulting in premature termination of protein translation explaining the shift in observed protein mass. Further investigations are required to identify the presence of premature proteins.

The fact that only 24 proteins were found to significantly change in all animals upon bromobenzene treatment seems to contradict the massive shift in the proteome pattern observed in the gel images (figure II.3). The application of strict quality criteria provides the main explanation for the limited identification of significantly changing spots. The low number of proteins that were found to significantly change using the proteomics approach, is also in contrast to the many genes that were found to be differentially expressed. This is partly explained by known non-linearity between mRNA synthesis and protein concentration and the temporal difference in responses. Moreover, technical limitations of the 2-dimensional gelelectrophoresis in terms of protein abundance, solubility and physico-chemical properties apparently prevented the detection of many proteins for which corresponding mRNA level changes were observed using transcriptomics. For about half of the proteins that were found to significantly change, the identification by mass spectrometry was not successful, either because we could not recover protein spots from the preparative gels or because an interpretable mass spectrum was not obtained. Indications for post-translational protein modifications were obtained from the detection of two forms of 4-hydroxyphenylpyruvic acid dioxygenase with similar mass but slightly different pI, and the carbamoyl phosphate synthase that was identified twice with differing pI and molecular mass.

II.5 Conclusions

The results presented here acknowledge the possibilities to study toxicology at the molecular level using transcriptomics and proteomics technologies. The confirmation of previously identified effects as well as the finding of genes and proteins not yet know to be involved in BB induced liver toxicity indicates that these technologies have the capability to expand insights in toxicology. Secondly, this evaluation stresses the difficulties and limitations in combining transcriptomics and proteomics results. While functional genomics technologies result in thousands of gene and protein measurements, many results can not be established with high confidence. Therefore, these methods require the application of strict quality filtering procedures, which we believe are of crucial importance for the outcome of the experiments. Due to the strict quality criteria, only a few proteins were identified as changing upon bromobenzene treatment, frustrating the finding of matching results from transcriptomics and proteomics analyses. Especially the proteomics technique using two-dimensional gelelectrophoresis and mass spectrometry for protein identification, does not easily allow the large scale identification of proteins involved in BB toxicity. However, new high throughput proteomics technologies (e.g. protein arrays) are being developed at high speed. While transcriptomics methods rapidly provide broad insights in cellular mechanisms, the determination of actual cellular protein and metabolite levels is required for the detailed elucidation of cellular processes in toxicity.

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Chapter III

Bromobenzene-induced hepatotoxicity at the transcriptome level

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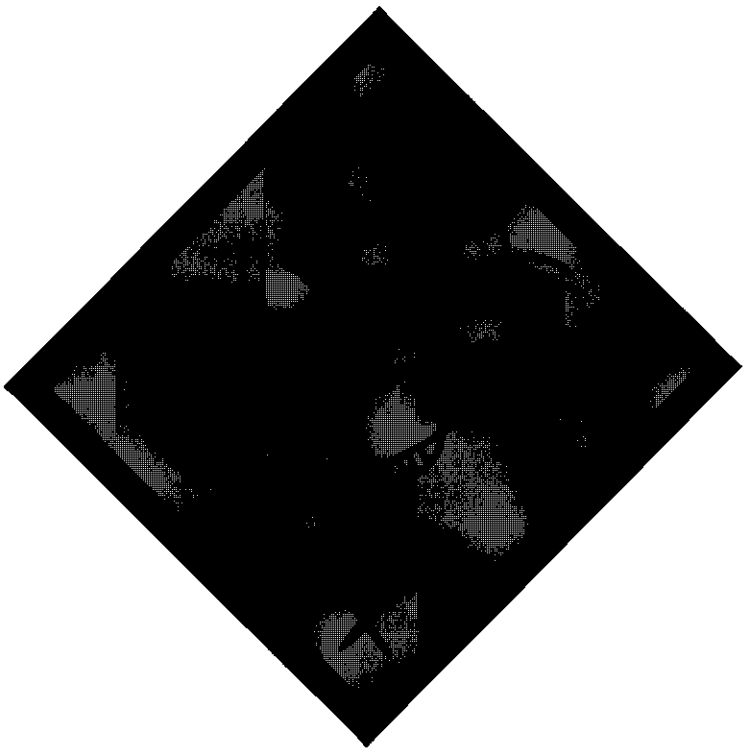

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Abstract

Rats were exposed to three levels of bromobenzene, sampled at 6, 24 and 48 hours, and liver gene expression profiles were determined to identify dose and time-related changes. Expression of many genes changed transiently, and dependent on the dose. Few changes were identified after 6h, but many genes were differentially expressed after 24 h, while after 48 h, only the high dose elicited large effects. Differentially expressed genes were involved in drug metabolism (upregulated GSTs, Ephx1, Nqo1, Mrps, downregulated Cyps, sulfotransferases), oxidative stress (induced HO-1, peroxiredoxin, ferritin), GSH depletion (induced Gclcl, Gsta, Gstm) the acute phase response, and in processes like cholesterol, fatty acid and protein metabolism, and intracellular signalling. Transcriptional regulation via the electrophile and sterol response elements seemed to mediate part of the response to bromobenzene. Recovery of the liver was suggested in response to BB by the altered expression of genes involved in protein synthesis and cytoskeleton rearrangement. Furthermore, after 48 h, rats in the mid dose group showed no toxicity, and gene expression patterns resembled the normal situation. For certain genes (e.g. Cyp4a, metallothioneins), intraday variation in expression levels was found, regardless of the treatment. Selected cDNA microarray measurements were confirmed using the specific and sensitive branched DNA signal amplification assay.

III.1 Introduction

Bromobenzene, an industrial solvent and an additive in motor oils, causes necrosis in the liver and kidney. The metabolism and toxicity of BB in (rat) liver have been studied in detail [74-78]. To enable excretion in urine, BB is subjected to biotransformation in the liver, and metabolites of BB are highly hepatotoxic while secondary metabolites of BB are highly nephrotoxic. Figure III.1 schematically depicts the biotransformation of BB in the liver. Upon entrance in the liver, BB is hydrolysed by Cyps, and inhibitors of Cyps were found to decrease the hepatotoxicity [101]. Cyp mediated epoxidation yields the highly electrophilic BB 3,4-epoxide. The irreversible binding of this very reactive metabolite to proteins like GST, L-FABP and carbonic anhydrase, is highly correlated to pathological effects [80]. The alternative, more stable, BB 2,3-epoxide was found to covalently bind soluble proteins like hemoglobin [102]. Phase II drug metabolising GSTs catalyse the sequestration of the reactive epoxides through conjugation to glutathione. The levels of GSH conjugates excreted in the bile correlated with the BB-dosage and the hepatotoxic effects [101]. The epoxides are also hydrolysed by the microsomal epoxide hydrolase and Cyps. The resulting bromophenols can be oxidised to hydroquinones, and conjugated to GSH. At high doses, conjugation to the metabolites depletes the hepatic GSH pool, and the intracellular protection against reactive oxygen species (ROS) and hazardous xenobiotic metabolites is lost. This may lead to a number of secondary events that damage the cell, like lipid peroxidation [97], ATP depletion, [98], [99], mitochondrial dysfunction, energy imbalance and altered intracellular calcium levels.

Recently, we reported a study where transcriptomics and proteomics were used to investigate BB-induced hepatotoxicity [2]. We explored the application of transcriptomics and proteomics in toxicology, and identified proteins that changed specifically 24 h after a single injection of BB. An increased abundance of proteins with lower molecular mass was observed, possibly indicating specific protein degradation. At the same time, a wide spectrum of genes in rat liver was differentially expressed. However, extensive toxicological examinations were omitted and only limited conclusions could be drawn on the mechanism of hepatotoxicity.

A new transcriptomics study was designed to investigate the sequence of events in time in hepatotoxicity, after oral exposure to BB, and to show the dose dependency of the observed effects at the transcriptome level. Measuring the expression of thousands of genes allowed more in-depth investigations in the hepatic changes at the molecular level in response to BB administration. Moreover, we expected to detect changes in gene expression at lower dose levels and earlier time points compared to the routine toxicity examinations.

Rats were given BB by oral gavage, at three dose levels and liver gene expression profiles were determined 6, 24 and 48 h later. Histopathology and clinical chemistry parameters in plasma were determined, as well as glutathione contents of the liver. Expression of several genes, selected based on the cDNA microarray results, was analysed in more detail using the bDNA assay.

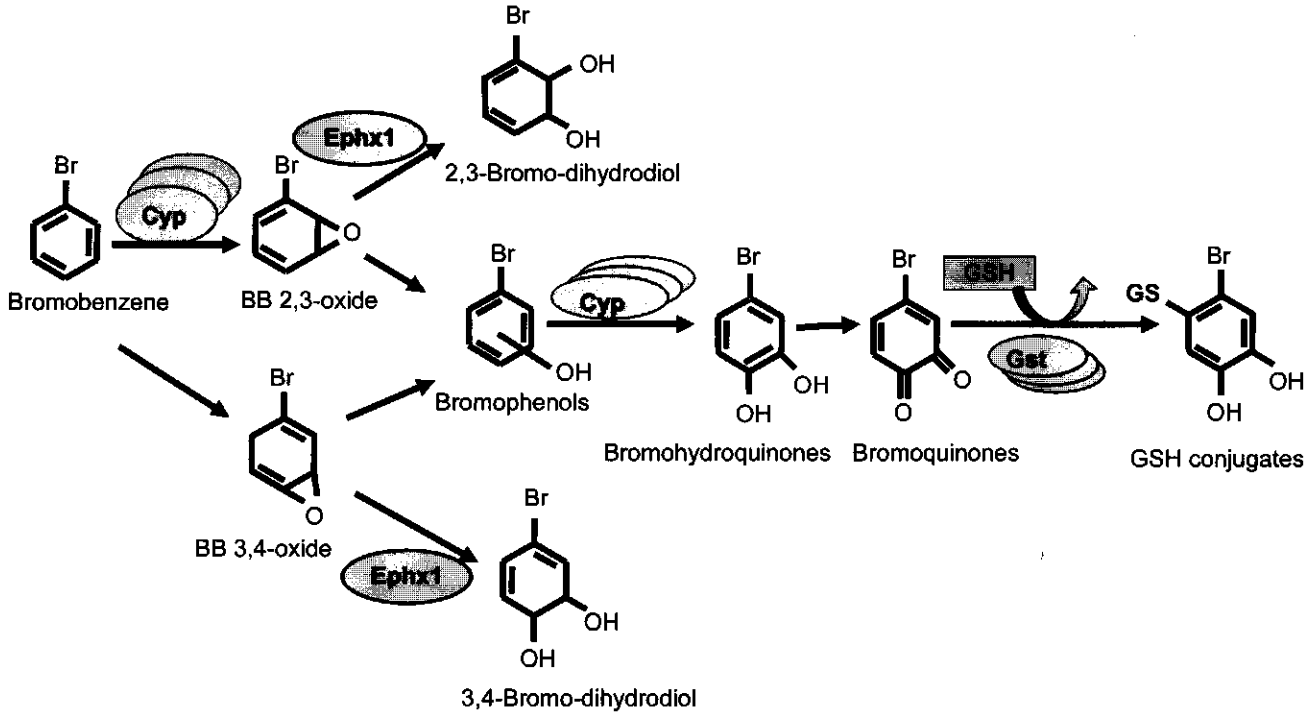

Figure III.1. *Mechanism of biotransformation of BB in rat liver.*
A schematic representation is given of the initial steps in biotransformation of bromobenzene in rat liver.
Cyp: cytochrome P450-enzyme; Ephx1: microsomal epoxide hydrolase; GSH: glutathione; Gst: glutathione-S-transferase

III.2 Materials and Methods

Animal treatment and sample preparation
Bromobenzene, 99%, was obtained from Sigma-Aldrich Chemie, GmbH (Steinheim, Germany). The LD_{50} of BB was reported to be approximately 20 mmol/kg body weight for male Crl:Cd rats (Haskell Laboratory for Toxicology and Industrial Medicine,1981, unreviewed). Three doses of BB (0.5, 2.0 and 5.0 mmol/kg body weight, dissolved in corn oil, 40% v/v) were administered by oral gavage to male Wistar rats (Charles River Deutschland, Sulzfeld, Germany), 10-12 weeks, body weight approximately 200 grams. Non-fasted rats were assigned to the groups by randomisation, and nine rats per dose group were treated with BB or corn oil, while an additional group contained the untreated controls (UT). Animals were kept under controlled conditions according to international guidelines and national legislation, regarding proper care and ethical use of animals. After dosage, rats received water and food *ad libitum,* but no food during the 6 or 16 hours before sacrifice. The rats were dosed at 9 a.m. on day 1. Three rats from each group were sacrificed after 6, 24 and 48 h, blood was collected in heparin tubes and livers were immediately dissected, frozen in liquid nitrogen, and stored at –80°C until further processing. A section of the liver was kept aside in formaline for pathological examination. Blood plasma was isolated for clinical chemistry. Livers were pulverised by mortar and pestle in liquid nitrogen.

GSH and clinical chemistry parameters
Glutathione ([GSH + GSSG] and [GSSG]) levels in liver homogenate were determined spectrophotometrically according to [81], and from these values, reduced glutathione, ([GSH]), levels were calculated. LDH, ALP, AST, ALT, bilirubin, cholesterol, phospholipids, triglycerides, glucose, GGT, creatin, albumin, urea and albumin to globulin (A/G) ratio in plasma were determined using a Hitachi-911 Bioanalyser, using Boehringer reagents, according to the manufacturer's protocols. One-way Analysis Of Variance (ANOVA) was used to assess the level of statistical significance of the changes.

RNA extraction
Total RNA was extracted from liver homogenate using Trizol (Life Technologies S.A., Merelbeke, Belgium) according to the manufacturer's protocol, and further purified using the RNEasy kit (Qiagen, Westburg B.V., Leusden, Netherlands), including a DNA digestion by RNAse-free DNAsel incubation. RNA was checked for purity and integrity by agarose gel electrophoresis and the concentration was determined spectrophotometrically.

cDNA Microarray preparation and labelling
cDNA microarray preparation was described previously, [2]. Briefly, about 3000 different sequence-verified rat cDNA fragments were arrayed on glass slides, and control spots were included. A typical labelling reaction was performed using 25 micrograms of total RNA, using the Cyscribe (Amersham Biosciences, Freiburg, Germany) fluorescence labelling kit. Cy3 or Cy5 fluorophore dUTP nucleotides were directly incorporated in the cDNA during the in vitro transcription reaction. RNA was degraded by hydrolysis in NaOH (30 min. at 37°C). Labelled cDNA was purified using an Autoseq G-50 (Amersham Biosciences, Freiburg, Germany) chromatography column. Hybridisation of labelled cDNA to the slides was performed as described before [2].

Image capture and analysis
Slides were scanned using a (Packard Biosciences) ScanArray Express confocal laser scanner, at wavelength 550nm (cy3 signal) and 650nm (cy5). TIFF images were analysed using Imagene (Biodiscovery Inc., USA.), and settings were applied to automatically flag weak or negative signals and spots with a non-homogenous signal. Excel (Microsoft Corporation, USA) and SAS (SAS, Cary, USA) were used to further process and analyse the data.

Transcriptomics experimental design
In order to compare all samples of individual rats to each other, and to other studies, an external reference sample was used. Thus, for each gene fragment, the amount of mRNA in the sample

relative to the amount in the reference was determined. The complete set of hybridisations was duplicated with swapping of the fluorophore incorporation in the sample and reference RNA. The value of this reference RNA has been described before [2]. A good correlation was found between the changes in gene expression determined in direct hybridisations and in indirect hybridisations using the reference.

DNA Microarray data preprocessing
After image analysis, the local background intensity was subtracted from the signal for each spot. Background intensities outside the cDNA spots were very low and homogeneous. Control spots and background fluorescence were used to determine a minimal signal to noise ratio threshold value of 1.5 for the two channels. Flagged spots and controls were excluded from further interpretation, as well as genes for which less than 75% of the microarrays yielded an acceptable signal. To account for technical variations introduced during labelling or hybridisation, data were normalised assuming that the majority of the transcripts was equally present in both samples. Since a relationship was found between the intensity of the signals and the variation in the ratio of gene, the lowess normalisation algorithm, according to [103], was applied in SAS software. This procedure fits the expression ratios to an intensity-dependent curve by locally weighted regression. After normalisation and logarithm transformation of the ratios tester/reference, a set of about 2700 rat cDNAs was used for further analysis. Excluded values were replaced by 0, and averages were calculated of the logarithms of fold changes between treatment groups.

DNA Microarray data analysis
Pair-wise comparisons of the expression ratios were made between samples of the different time and dose groups and controls. In these comparisons, untreated and corn oil control samples were considered as one control group, since preliminary analyses revealed only minor changes induced by single oral corn oil dosage. Statistics (two-sided, unpaired t-tests) was applied assuming unequal variance and changes were considered significant if the tests resulted in a p-value less than 0.01. In the tables, p-values were denoted as follows: *** : $p < 0.001$; ** : $p < 0.01$. The genes significantly differentially expressed upon treatment were explored in the context of biological mechanisms and pathways. Genes were categorised based on biological processes using literature and gene information databases.

Gene expression measurement by branched DNA signal amplification assay
mRNA levels of Ephx1, HO-1, Nqo1, Mt-1, Gclc, Cyp2b1/2, Cyp4a2/3, Mrp1, Mrp2 and Mrp3 and Gapdh were analysed for all samples individually by the bDNA assay using probes specific to each transcript (Quantigene HV10 kits, Genospectra, www.genospectra.com) as described in [104]; [105]. Oligonucleotide probe sets that detect Nqo1, Cyp2b1/2, Cyp4a2/3, Mt-1, Mrp1, Mrp2, and Mrp3 were previously described [106]; [104] ; [107] Oligonucleotide probe sets for Ephx1, HO-1 and Gclc are described in Table III. 5 (supplementary material)

III.3 Results

Changes in the liver transcriptome were related to classical toxicological parameters, which showed toxicity only after the highest of three dose levels of BB. Dose-dependent effects of BB at the transcriptome level were analysed per time point. Additionally, we report intraday variation in gene expression regardless of the treatment. The bDNA assay was used to further investigate changes highlighted by the DNA microarrays.

(Histo)pathology

No significant changes in body weights or macroscopic changes were seen. Neither the livers of the controls nor of the low or mid dose groups displayed pathological aberrations. The livers isolated 24 h after a high dose of BB revealed a patchy appearance and gross lesions, while the livers of rats sacrificed 48 h after a high dose had focal discoloration. Six h after dosing of mid and high levels of BB, relative liver weights were around 10% lower than the controls (ANOVA + Dunnett's test, $p < 0.05$ for high and $p < 0.01$ for mid). At 24 or 48 h, controls as well as low dosed rats had relative liver weights of 75-85% of the rats sacrificed after 6 h. In all the high BB treated rats, a significant increase to around 130% of vehicle controls ($p < 0.01$) was observed. Microscopic examination of the livers showed slight presence of mononuclear cell aggregates and/or necrotic hepatocytes in several rats regardless of the treatment. After 24 and 48 h, pronounced centrilobular necrosis was found in all rats of the high dose groups, with interindividual variation from very slight to very severe necrosis.

Clinical chemistry

Clinical chemistry parameters in plasma are represented in Figure III.2. Bilirubin levels (panel A) in the mid dose group were increased after 24 h, but not after 48 h. In the high dose group, bilirubin levels were slightly up at 6 h, and highly elevated after 24 h and 48 h. Only upon high dose, the rats showed highly elevated ALAT and ASAT levels after 24 h and 48 h (panels B and C). Plasma lactate dehydrogenase (LDH, not shown) activity was increased 100 fold 24 h after the high dose, but showed normal levels at 48 h. Alkaline phosphatase (ALP, panel D) activity in plasma gradually increased with dose at 24 h (not significant), and was elevated 48 h after the high dose. Plasma glucose (panel E) decreased equally by mid and high dose after 24 and 48 h. Cholesterol (n.s.) and phospholipids levels (panel F) in the high dose group were increased at 6 h and remained elevated. The creatin content decreased upon the high dose after 6 h (n.s.). Regardless of the treatment, urea levels were higher and triglyceride levels lower at 24 and 48 h compared to 6 h. No significant changes were observed in plasma levels of gamma-glutamyl transpeptidase, total protein, albumin, and the albumin to globulin ratio (A/G).

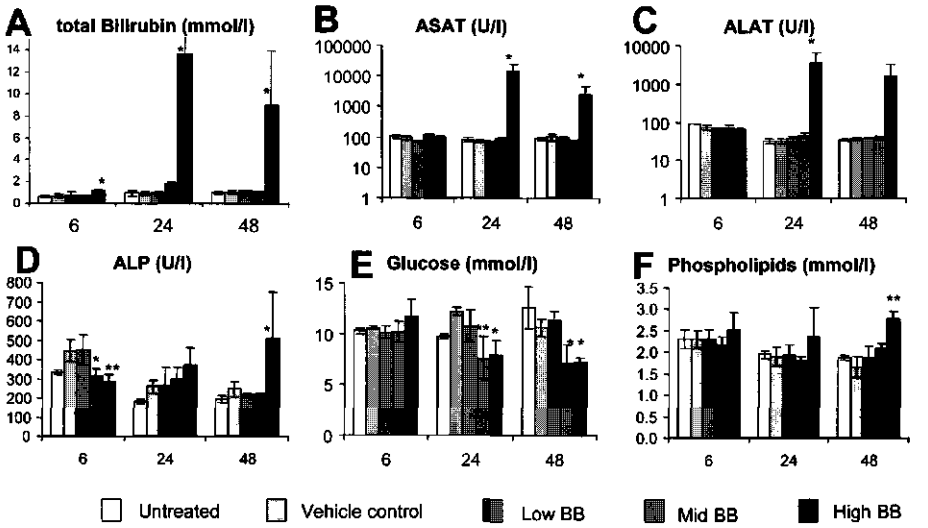

Figure III.2. *Clinical chemistry parameters in blood plasma*
Measurements of clinical chemistry parameters in plasma. Levels are depicted of bilirubin (panel A), ASAT (panel B) and ALAT (panel C), alkaline phosphatase (ALP, panel D), glucose (panel E), phospholipids (F). Average levels of the treatment groups ± SD are represented. Statistical significance is denoted with ** p-value < 0.01; *** p-value < 0.001.

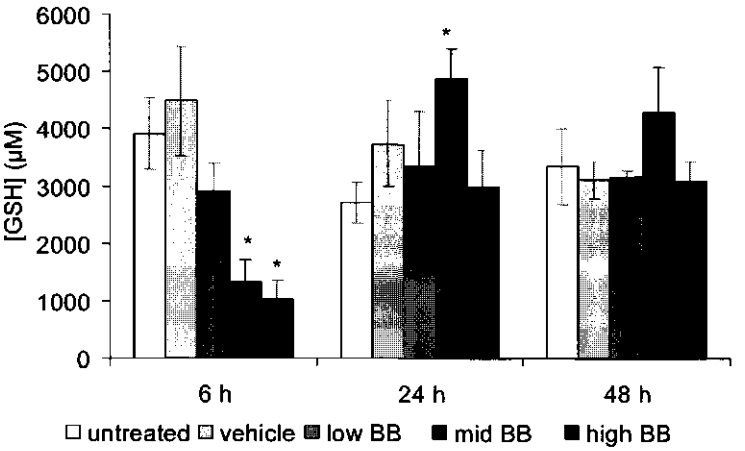

Figure III.3. *Hepatic intracellular glutathione levels*
Intracellular reduced glutathione, [GSH], levels were determined in rat liver homogenate 6, 24 and 48 hours after a single dosage of bromobenzene at low, mid or high dose. Average levels of the treatment groups ± SD are represented. Statistical significance is denoted with * for a p-value < 0.005

Glutathione depletion

Hepatic reduced GSH levels, after correction for oxidised disulfide of glutathione (GSSG), are shown in Figure III.3. An intraday variation in the concentration of GSH was observed in control rats. More GSH was present at 6 h compared to the 24 h or 48 h time point, The low dose of BB caused a slight decrease of GSH levels after 6 h, whereas both the mid and high BB doses depleted GSH to ~25% of control levels. After 24 h, GSH levels in the mid dose group increased significantly to ~125% of controls, and remained elevated 48 h after dosage.

Gene expression

Pair-wise comparisons of the gene expression levels were made between treated and control groups. After 6 h, only few changes could be identified, whereas many genes changed in expression 24 and 48 h after dosage. Overlap of the genes differentially expressed upon BB treatment at all time points was observed, although many genes were altered with statistical significance only at one time point. The individual gene expression profiles of all the BB-treated rats sacrificed after 24 h were recorded in duplicate. Many genes were regulated after a high (176 genes) or mid dose of BB (41 genes), whereas a low dose induced changes that were significant in only 6 genes ($p < 0.01$). The majority of the genes significantly regulated 24 h after the mid dose were also regulated after the high dose, whereas most of the genes that were changed upon the low dose were altered uniquely in this group.

6 hours

Table III.1 lists the genes that were significantly differentially expressed after 6 h in the BB dosed rats. A marked (2 - 8 fold) increase in gene expression was observed for several metallothioneins (Mt). Both the flavin–containing mono-oxygenases Fmo1 and Fmo3 were upregulated by the high dose, as well as cathepsin L and ASAT. The enzymes cysteine dioxygenase 1 and betaine homocysteine methyltransferase were upregulated. A twofold upregulation was found for mRNA encoding Rho-interacting protein 3. Genes downregulated by the high dose include LDH B, tubulin, and cholesterol metabolism enzymes farnesyl diphosphate synthase and farnesyl diphosphate farnesyl transferase 1, 'sterol-C4-methyl oxidase-like', HMG-CoA synthase 1. Several genes, HO-1, Timp1, Afar and serine protease 15, changed significantly with the mid or the low dose after 6 h but not with the high dose. Three genes were down regulated by both the mid and high dose, namely the 'EST similar to GSTP', Hsp70 and calpain.

Table III.1 *Genes differentially expressed by bromobenzene after 6 hours*

Category	Gene name	Acc#	low	mid	high
Ox.stress	Heme oxygenase (Ho-1)	AA874884	0.76	2.32 **	0.57
Ox.stress	tissue inhibitor of metalloproteinase 1	AA957593	1.14	1.92 **	0.51
Ox.stress	Metallothionein (Mt)	AA859399	0.98	3.52	3.01 ***
Ox.stress	Metallothionein (Mt)	AA900218	0.71	3.06	2.42 ***
Ox.stress	High sim to Metallothionein-II	AA924281	0.91	1.88	2.15 ***
Ox.stress	Weak sim to Metallothionein-I	AA819204	0.43	1.04	1.19 **
AcutePhase?	nuclear protein 1	AI070183	0.22	-0.42	-0.58 **
Aminoacid	Aspartate aminotransferase (Asat)	AA900928	-0.28	-0.32	0.82 **
Cholest.	farnesyl diphosphate farnesyl transferase 1	AA818927	-0.34	-0.40	-0.71 **
Cholest.	3-HMG-CoA synthase 1	AA924800	-0.26	-0.21	-0.83 **
Cholest.	farensyl diphosphate synthase	AA859192	-0.30	-0.79	-0.87 **
Cholest.?	sterol-C4-methyl oxidase-like	AA859607	-0.21 **	-0.08	-0.31 **
Cysteine	cytosolic cysteine dioxygenase 1	AA818579	-0.30	0.13	0.67 **
Cysteine	betaine-homocysteine methyltransferase (Bhmt)	AA901407	0.08	-0.07	0.61
Biotransf	flavin-containing monooxygenase 3	AA964011	-0.03	-0.18	0.87 **
Biotransf	Flavin-containing monooxygenase 1	AA860001	-0.03	0.14	0.46 **
Biotransf	cytochrome P450 4A3 (Cyp4A3)	AA924591	0.33	0.38 **	0.19
Biotransf	cytochrome P450, 2b19 (Cyp2B19)	AA818412	-0.63 **	-0.21	0.08
Biotransf	sulfotransferase family, 2A1	AA819605	-0.87 **	-0.43	-0.33
Biotransf;	Glutathione S-transferase 1 (Gstt1)	AI044236	-0.13	-0.26 **	-0.03
Biotransf;	Weak sim to GST P (Gst 7-7)	AA819810	-0.40	-0.64 **	-0.56 **
Biotransf	aflatoxin B1 aldehyde reductase (Afar)	AA923966	1.09	1.23 **	0.07
Heat shock	heat shock 70kD protein 5 (Hsp70)	AI453996	-0.27	-0.86 **	-0.70 **
Immuno	ig delta heavy chain constant region	AA964201	0.09	0.15	0.26 **
Immuno	MHC class I	AA818887	-0.28	-0.22	-0.53 **
LDH	lactate dehydrogenase B (Ldh)	AA819821	-0.32	-0.27	-0.59 **
Proteolysis	cathepsin L	NA	-0.18	-0.08	0.59 **
Proteolysis	protease, serine, 15	AI070052	0.53 **	0.39	0.24
Proteolysis	sim to ubiquitin conjugating enzyme	AA818770	0.28 **	0.10	-0.01
Proteolysis	High sim to leucine aminopeptidase	AA858780	-0.35	-0.41	-0.48 **
Proteolysis	calpain 1	AA901002	0.06	-0.56 **	-0.34 **
Proteolysis?	ubiquitin D	AI030354	-0.32	-0.34	-0.44 **
Ribosome	ribosomal protein L22	AA924274	-0.16	-0.18	0.64 **
Ribosome	ribosomal protein L28	NA	0.30	0.44 **	0.13
Ribosome	ribosomal protein L35	AA925200	-0.05	0.38	-0.30 **
Structure	Rho interacting protein 3	AA924848	-0.13	0.09	1.01 **
Structure	Unconventional myosin from rat 3	AA818082	-0.77 **	-0.27	0.10
Structure	actinin alpha 4	AI712704	-0.21	-0.21 **	-0.11
Structure	beta-tubulin T beta15	AA899219	-0.31	-0.20	-0.56 **
Transcr. factor	Transforming growth factor beta stimulated clone 22	AI137902	0.10	-0.64 **	-0.30
Transport	vitamin D-binding protein	AA818706	0.24	0.35 **	-0.03
signal transd.	14-3-3- protein Ywhaq	AA858957	-0.02	-0.33	-0.34 **
Other	S6 kinase	AA900032	0.10	-0.12	0.36 **
Other	sim to Suc-CoA:3-ketoacid-coA transf.	AA819087	-0.46	-0.23	-0.72 **

24 hours

Table III.2, in supplementary material, lists the subset of genes that changed significantly 24 h after BB, in the high dosed rats. Genes were categorised according to biological processes. Amongst many others, Gsta and Gstm, Ephx1, Afar, ferritins, peroxiredoxin, transketolase1, Gapdh, Nqo1, proteasome subunit alpha1, and ALP were markedly elevated upon mid and high dose. The genes encoding 'EST similar to human phosphodiesterase (cAMP specific)' and the alpha(1)-inhibitor 3 were markedly down regulated by all doses of BB, while the glucocorticoid receptor was mildly but significantly downregulated by all doses (not shown). Decreased upon mid and high dose were the genes encoding cysteine dioxygenase, asialoglycoprotein receptor 2, 14-3-3 protein (Ywhaz), 'EST similar to GSTP', sulfotransferase 2 and the transcription factor 'core promoter element binding protein'(C/EBP).

48 hours

The differential expression of genes 48 h after the high concentration of BB was similar to the expression pattern after 24 h (Table III. 3) Many of these 175 genes were also differentially expressed after 24 h, although frequently the difference was less marked and not statistically significant. Only two genes, interferon-inducible transmembrane protein and steroid sulfatase were found to be subtly but significantly differentially expressed at the low BB dose. The mid dose slightly induced three genes, also induced by the high dose.

Intraday variation

The control rats were used to analyse the intraday variation in liver gene expression as well as in other parameters. Intraday differences were observed in GSH content and relative liver weights as well as in levels of parameters in plasma. Triglyceride levels were lower at 24 h (and 48 h) than at 6 h. This was also the case for ALP, phospholipids, cholesterol and urea Also at the gene expression level, the 6 h time point was found to be clearly distinct from the 24 and 48 h time points. Profiles obtained from untreated rats were very similar to those obtained from corn oil treated rats. No genes were recognised with a marked change in expression between 24 and 48 h in the controls. Table III. 4 shows genes which' expression changed significantly from 6 h to 24 h in controls. The most distinctly higher expressed genes were metallothioneins. Moreover, various Cyp isozymes (Cyp4A1 and Cyp4A2/3), arginosuccinate lyase, cathepsin L and various genes involved in fatty acid metabolism were higher expressed at 24 h than at the 6 h time point. Genes which' expression was lower after 24 h compared to 6 h included several GSTs, transferrin, tubulin, lysyl hydroxylase and GSH peroxidase.

Table III. 4. *Gene expression changes comparing 6 and 24 h time points*

The subset of genes that were significantly ($p < 0.01$) differentially expressed between the 6 h and 24 h time point in control rats. Genes were categorised according to biological mechanism or pathways. The log (base 2) average fold change and the p-value for the three dose levels are listed. A treshold was chosen for display reasons which was a log (base2) fold change > 0.5 or < -0.5 in any of the dose groups compared to the controls. Statistical level of significance: **: $p < 0.01$, ***: $p < 0.001$. ACC#: Genbank Accession number, metab: metabolism

Category	Gene name	ACC #	24h - 6h	
Acute Phase; Ox.stress	Metallothionein (Mt)	AA859399	3.24	***
Acute phase; Ox.stress	Metallothionein (Mt)	AA900218	2.65	**
Acute phase; Ox.stress	Transferrin	AA858975	-0.69	**
Aminoacid	argininosuccinate lyase	AA818673	1.05	**
Cholesterol	farnesyl diphosphate synthase	AA859192	-1.06	**
Drug metab	cytochrome P450, 4a10 (Cyp4a10)	AA956787	1.50	**
Drug metab	cytochrome P450 4A3 (Cyp4a3)	AA924591	1.32	***
Drug metab; GSH	GST, soluble,class Mu ?; Gst Y(b)	AA998734	-0.73	**
Drug metab; GSH	Weak sim to GST P (Gst 7-7) (pi)	AA819810	-1.08	**
Drug metab; GSH	Weak sim to GST P (Gst 7-7) (pi)	AA819810	-1.14	***
Drug metab; GSH	glutathione S-transferase, alpha 1 Gsta	AA818339	-1.21	**
Drug metab; GSH	Glutathione peroxidase 1 (Gpx1)	AA964788	-1.32	**
Fatty acid	dodecenoyl-coenzyme A delta isomerase Dci	AA965078	1.69	**
Fatty acid	delta3, delta2-enoyl-CoA isomerase Dci	AA997009	1.30	***
Fatty acid	2,4-dienoyl CoA reductase 1, mit.	AA875267	0.64	**
Fatty acid	cytosolic acyl-CoA thioesterase 1	AA925003	0.50	**
Proteolysis	cathepsin L	NA	0.90	***
Proteolysis	Cathepsin L	AA859498	0.77	**
Structure	beta-tubulin T beta15	AA899219	-0.65	***
Other	enoyl coenzyme A hydratase 1	AA926032	1.05	**
Other	isocitrate dehydrogenase 1	AA925731	-0.51	**
Other	cytochrome c oxidase subunit Vb	AA955550	-0.64	**
Other	lysine hydroxylase	AI045272	-1.32	**

Confirmation of gene expression changes

cDNA microarrays results for selected genes were analysed for all rat samples using the bDNA assay (Figure III.4). This method was shown to very specifically determine mRNA levels in a wide concentration range. The gene expression levels for Ephx1, Gapdh, HO-1, Mt-1, Nqo1 and Cyps in the bDNA assay largely overlapped with the cDNA microarray results. For example, between both methods, a coefficient of correlation of 0.94, 0.89 and 0.86 was found for the individual rats' levels of Ephx1, HO-1 and Mt-1, respectively. Also, genes were analysed that were not (conclusively) measured in the microarrays. We hypothesised these genes (Mrp1, Mrp2 or Mrp3, Gclc) could be modulated based on other changes observed using the microarrays, for instance indicating EpRE-mediated transcriptional regulation. Gapdh, which is frequently regarded as a so-called housekeeping gene with stable expression levels, was measured using both the microarrays and the bDNA assay. By both methods, Gapdh was found to be upregulated more than two-fold at high dose levels after 24 h.

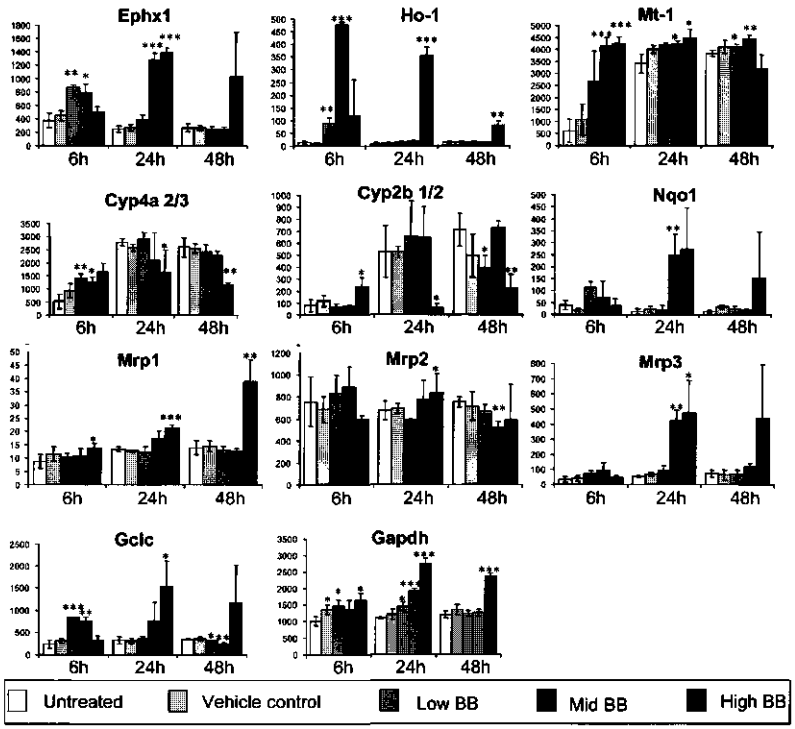

Figure III.4. *Hepatic gene expression levels as measured by the Branched DNA signal amplification assay.*

Average levels of the treatment groups ± SD are represented. White bars represent samples taken 6 h after dosage, grey bars 24 h and black bars represent samples taken 48 h after dosage. UT: untreated; CO: corn oil controls; L, M and H denote the low, mid and high dose of bromobenzene. Statistical significance : ** :p-value < 0.01; *** : p-value < 0.001.

III.4 Discussion

The dose and time related effects of acute hepatotoxicity induced by BB were analysed at the gene expression level. Hepatic centrilobular necrosis was found after 24 h in rats at the high dose, but not at lower doses. Also clinical chemistry parameters detected hepatocellular damage only after 24 h in the high dose group. Significant changes in expression of genes upon the high, mid and even the low dose were identified, especially after 24 h. The large part of the genes identified to change significantly upon the high dose also responded to the mid dose, although frequently not as pronounced or not with the same statistical significance. Thus, a dose-relationship can be observed in transcriptomics experiments, both in the number of genes that change and the magnitude of the changes. In time, the mid dose elicited most effects after 24 h, while after 48 h, hardly any change persisted. In contrast, in the high dose group, those effects identified after 24 h persisted after 48 h.
Paradoxically, several genes including Ephx1, Afar, HO-1, Timp1 were markedly upregulated by the low and mid dose at 6 h, but not by the high dose. At later stages, these genes were highly upregulated by BB in a dose-dependent manner. The effects measured by cDNA microarrays were confirmed using the bDNA assay. The initial lack of response with the high dose contradicts the dose-dependency usually assumed in toxicology. No explanation was found for this phenomenon, and no such observations were found in literature.

Pathways and mechanisms
Genes with statistically significant differential expression upon BB administration were categorised according to biological processes in the cell, putatively relevant in BB-induced hepatotoxicity. The most relevant changes in BB-induced hepatotoxicity were schematically displayed in Figure III.5.

Drug metabolism
The strong induction of transcripts encoding Gsta and Gstm stresses the importance of GSH conjugation in the biotransformation of BB. The upregulation of Ephx1 mRNA levels by BB is coherent with its role; the hydrolysis of epoxide BB intermediates, enabling their excretion. The epoxides are amongst the most reactive BB-metabolites and their hydrolysis is crucial in the hepatic detoxification. The Ephx1 is also induced by xenobiotics like phenobarbital, *trans*-stilbene oxide and Aroclor 1254 [85]. BDNA assays revealed about two-fold increased expression levels as early as 6 h after the low dose. Mid or high doses elevated Ephx1 expression after 24 h, while after 48 h, Ephx1 was pronouncedly upregulated only in the high dose rats. Similar to Ephx1, induction of Nqo1 was identified using the bDNA assays. Nqo1 is an enzyme with a role in protection of cells from oxidative stress, cytotoxicity, and mutagenicity of quinones, which are also formed in the biotransformation of BB. The induced Afar, and aldo-ketoreductases exert putative roles in metabolism of xenobiotics and products from lipid peroxidation like 4-hydroxy-2-nonenal. The early induction of Fmos has possible implications in the biotransformation of BB as well. Fmos have a broad substrate specificity and their functions partly overlap with Cyps. Induction of Fmo1 was found in rat liver by the polycyclic aromatic hydrocarbon, 3-methylcholanthrene [108]. Different Cyp (2B1/2, 2E1, 2A2C39, 4A2/3, 4A10, 2C12, 2C23) and sulfotransferases were down regulated and remained lower after 24 h. The bDNA assay confirmed specific changes in expression levels of the Cyp2B 1/2 and Cyp4A 2/3 isoforms. At 6 h, BB slightly increased Cyp2B and Cyp4A expression, while after 24 and 48 h, expression was reduced upon high BB treatment.

Moreover, intraday variation in expression was observed in the controls. The decreased gene expression of Cyps could diminish the bioactivation of BB to harmful metabolites. Expression of the multidrug resistance proteins Mrp1, Mrp2 and Mrp3 was measured with the bDNA assay. The change in expression of Mrp1, Mrp2 or Mrp3 was not measured in the microarrays, but Mrp3 induction was hypothesised from the suggested EpRE-mediated induction of several genes. A pronounced induction of Mrp3 gene expression was observed 24 h after high BB treatment, whereas the Mrp2 expression did not change. Mrp1 expression levels were very low in the liver, but an induction was observed by high BB after 24 h. Substrates for Mrp-facilitated export across the membranes include glucuronide, sulfate and GSH conjuates. The induction of Mrps by BB could enhance the biliary excretion of conjugated BB metabolites, as found for acetaminophen metabolites [105] ; [109]. The concurrent increased hydrolysis of the epoxides, GSH conjugation and clearance of the harmful BB metabolites may provide the liver with an effective mechanism of detoxification.

GSH metabolism

GSH is believed to be of crucial importance in the detoxification of xenobiotics like BB. A dose-dependent lowering of the liver GSH levels was observed 6 h after BB administration. Compared to our previous study with intraperitoneal injection of BB [2], total GSH depletion was not observed in this study, 24 h after an oral administration. On the contrary, after 24 h GSH concentration was higher upon BB administration than in the controls. The increased level of GSH after the initial depletion has been suggested as a recovery mechanism [110]. Also, a significant upregulation of Gsta and Gstm was found after 24 h even by the low dose. The rate-limiting enzyme in GSH synthesis is Gclc, transcriptionally regulated by EpRE. Using the bDNA assay, we detected a pronounced upregulation of Gclc as early as 6 h after the low dose (Figure III.6). Recently, we also found GSH synthase protein to be induced by BB [2]. The upregulation of the enzymes in GSH metabolism can be explained as an adaptive response to restore the depleted GSH levels in liver.

Oxidative stress

Highly reactive metabolites such as formed from BB can induce oxidative stress. Additionally, the depletion of GSH diminishes the intracellular protective mechanism against ROS and electrophilic metabolites The early induction of key markers, the mRNA levels of HO-1 and Timp1 suggests the induction of oxidative stress by BB After 24 h, HO-1 mRNA levels were elevated only in the high dose group, while returned to normal in the mid dose group. HO-1 catalyses the degradation of heme to CO_2 and biliverdin, which is subsequently catabolised to bilirubin. Bilirubin may serve as an intracellular antioxidant. In line with this, we observed bilirubin levels in plasma to be slightly increased in the high dose group at 6 h, while largely increased after 24 h. Also peroxiredoxin 1 and the ferritin light and heavy subunit transcripts were elevated significantly by BB. Ferritins sequester free iron molecules thus preventing formation of ROS, hydroxyl radicals, through the Fenton reaction. Peroxiredoxins are antioxidant enzymes with a role in signal transduction. Metallothionein transcripts were highly induced at 6 h after all doses of BB. At 24 and 48 h an elevation of the Mt mRNA levels was observed independent of the treatment. Metallothioneins are cysteine-rich proteins that function in the sequestration of the metals Cu^{2+}, Cd^{2+}, Zn^{2+}, while also ROS can be scavenged. The BB-induced rapid increase of Mt mRNA corresponds with reported 40% increased protein concentrations in the liver and kidneys 6 h after BB. [111] and [112]. The protein Vdup1 was induced and has been reported to interact with thioredoxin and to be

associated with oxidative stress. [113]. GSH peroxidase, selenoprotein P and synaptojanin 2, genes possibly related to oxidative stress, were down regulated. Gapdh was induced more than two-fold 24 after the high dose of BB. Gapdh has been reported to be regulated by hypoxia and induced by insulin and glutamine. Responsive elements for hypoxia-inducible factor, and C/EBP have been identified in the Gapdh gene promotor [114,115]; [116] The induction of Gapdh might be in concordance with its function in glycolysis, upregulated to meet the energy requirements of the regenerating liver.

Acute phase response
The acute phase response is elicited by various types of stress like mechanical damage and inflammation [92]; [93]. Especially in the liver, changes in many genes and proteins provide a protective response and re-establish cellular homeostasis. We previously identified that the acute phase response was elicited upon an intraperitoneal administration of BB [2]. Present experiments showed that also upon oral gavage, BB was able to elicit changes in transcript levels of many acute phase proteins in liver. Orosomucoid 1 (former AGP), cytokeratin-18, apolipoprotein A1 were induced. Negative acute phase transcripts including alpha-1-inhibitor 3 and pre-alpha-inhibitor heavy chain 3, serine protease inhibitor, complement component 1 and 4, L-FABP 1 and fibrinogen B were down regulated by BB.

Fatty acid & cholesterol metabolism
Plasma levels of cholesterol increased upon high dose after 6 h and remained elevated at 24 and 48 h. Several enzymes involved in cholesterol metabolism were significantly and dose-dependently down regulated after 6 h by BB. Cholesterol is biosynthesised from acetyl-CoA, the product of fatty acid degradation, and enzymes in both pathways are down regulated by BB, including HMG-CoA synthase, Acetyl-CoA acetyltransferase 1, hydroxylacyl-CoA dehydrogenase, acyl-coA oxidase, trifunctional protein. Many of the genes coordinately downregulated are transcriptionally induced through binding of the sterol responsive element binding protein (SREBP) to a sterol-responsive element in the upstream DNA sequence of those genes. [117], [118]. Cholesterol levels play a role in the regulation of the SREBP pathway. The downregulation of the fatty acid and cholesterol metabolism could be due to the requirement of energy for these processes. In this situation of distress, the cell might have to dedicate all energy supplies to cope with the toxicity induced by BB, and restore homeostasis. Energy-requiring processes should be down regulated, while energy-generating processes are upregulated. In line with this, the decrease of plasma glucose levels could be ascribed to increased catabolism of glucose in the glycolysis.

Protein synthesis & proteolysis
Genes involved in protein synthesis, including many ribosomal subunits, eukaryotic initiation factors and elongation factor, were over-expressed one day after dosage. Furthermore, an induction was observed for several components of the proteasome and the proteolytic enzyme cathepsin L. Dose and time related changes in gene expression were observed for serine protease, dipeptidyl peptidase, polyubiquitin and calpain. The histopathologically observed slight nucleolar enlargement and the mitotic increase in the BB-treated livers correspond with the induction of proteins required for transcription and translation.

Structure & cytoskeleton

Genes encoding cell-structure proteins increased upon the high dose of BB. Stongly upregulated were actin and 'weakly similar to pervin', a protein with high homology to a human cytoskeleton-interacting protein. Also actinin, cortactin, 'EST highly similar to actin', keratin, dynein, tubulins and others were differentially expressed. Thymosin beta-4 is an actin binding protein and is upregulated 48 h after a high dose of BB. Rho-interacting protein is involved in cytoskeleton rearrangement, and increases after 6 h. Oxidative modifications of the microfilaments are suggested to cause cytotoxicity in the form of blebs on plasma membranes, when polymerised actin is disrupted by oxidation of its sulfhydryl groups. Upon rupture of the blebs, cellular ion gradients and intracellular components are lost, leading to necrotic cell death. The change of cellular calcium (Ca^{2+}) levels may play a role in the cytotoxicity. Increasing Ca^{2+} levels promote dissociation of actin from α-actinin, and activation of the calpain protease, which cleaves actin-binding proteins. When the anchoring of the cytoskeleton to the plasma membrane is disturbed, membrane blebbing may occur [119]; [120]. BB caused down regulation of calpain gene expression after 6 h. The induction of both the protein synthesis and the cell-structure genes suggests that enhanced protein synthesis and/or proliferation occur, which aids in hepatic tissue remodelling and recovery after BB-induced hepatocellular injury. Corroborating evidence is found in the histopathological observation of a mitotic increase and nucleolar enlargement in the hepatocytes upon high BB dosage.

Recovery

The gene expression profiles obtained from livers isolated 48 h after a mid BB dose resembled the profiles of the controls and the low dose livers. We suggest that the mid dose still effectuates a marked response, especially detectable 24 h after oral ingestion of BB, but is not high enough to induce irreversible damage detectable by histopathology or clinical chemistry. This suggests that the rats in the mid dose group did not suffer, or recovered from the toxic stimulus. From the time-series, it was clear that most pronounced changes in gene expression were observed 24 h after BB administration. Previously, [121] suggested that irreversible changes occurred after 24 h, when the limited centrilobular lesions progressed to a more widespread pattern, and DMSO could no longer attenuate the BB-induced necrosis.

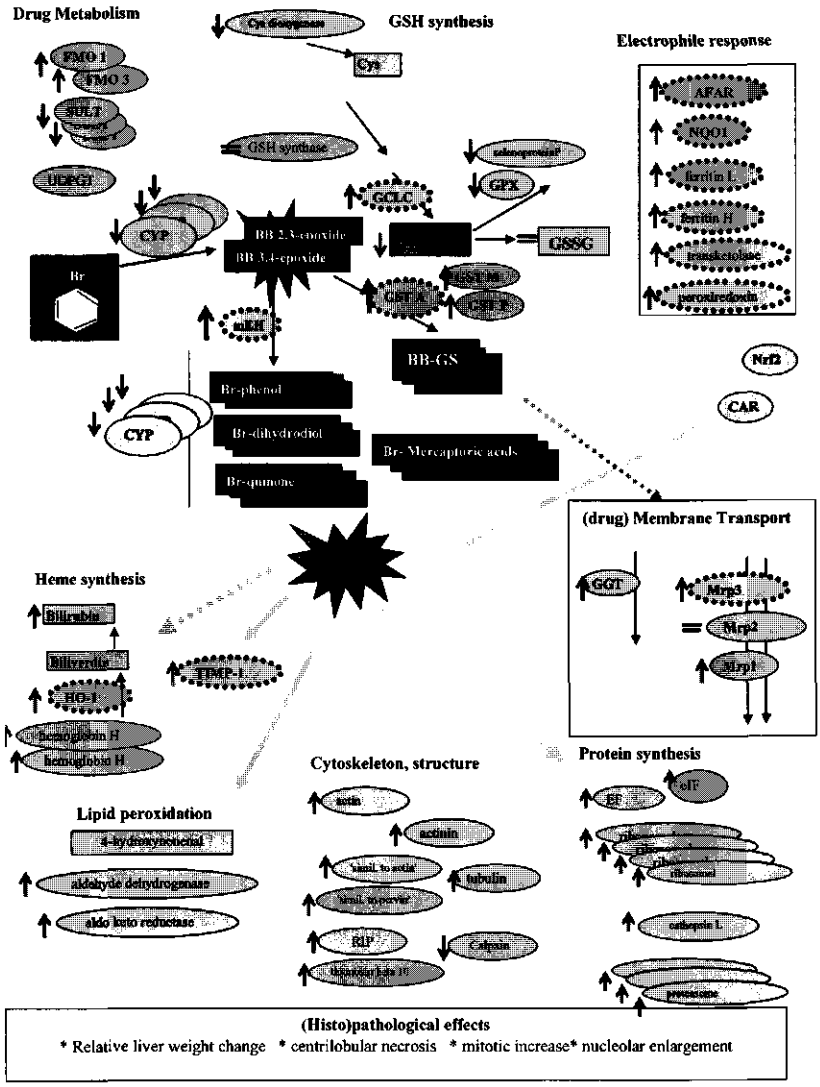

Figure III.5. *Schematic representation of the most prominent changes in bromobenzene-induced hepatotoxicity.*
The biotransformation of BB in rat liver, and biological processes relevant to the hepatic response to BB are shown. Blue rectangular boxes represent the metabolites formed from BB in biotransformation. Filled circular boxes represent genes, grey rectangular boxes represent metabolites or small molecules. A red arrow is pointing up when the gene is upregulated by BB and a green arrow is pointing down when a gene is downregulated by BB. Circular boxes with dashed lines are shown for the genes known to be transcriptionally regulated by an electrophile response element (EpRE). Oxidative stress induced by the reactive metabolites of BB plays a central role in the response at the gene expression level.

Coordinate expression mediated by the electrophile response element
The coordinate induction of HO-1, ferritins as well as Gsta, and Nqo1 by BB is consistent with the reported presence of an electrophile response element (EpRE), formerly named antioxidant response element (ARE), in those genes [122]; [86]; [87]. Rat Gsta, Nqo1 and ferritin were known to be transcriptionally regulated by binding of Nrf2 to this EpRE. More Nrf2-regulated genes were identified in mice upon induction by the isothiocyanate sulforaphane [123]. These included Afar, glucose-6-phosphate dehydrogenase, carboxylesterase, transketolase, and aldehyde dehydrogenase. In our studies in rats, BB induced HO-1, ferritins, Gsta, Gstm, and Nqo1, concurrently with Timp1, Afar, Gstm, peroxiredoxin1, aldo-keto reductase, transketolase, and also Mrp3. The rat Mrp3 was recently found to be induced by CAR and EpRE activators in liver. [104] The presence of the EpRE in rat Afar, as suggested by our data, was confirmed recently [124].

Intraday variation in gene expression
Control rats sacrificed 6 h after the start of the study had higher relative liver weights and also the levels of GSH differed. Urea, triglycerides and phospholipids levels in plasma varied on an intraday basis. These changes were accompanied by altered liver gene expression. Intraday variation of GSH levels (and of other plasma parameters) might not be a negligible effect on the outcome of pharmacology and toxicity studies. Our results show that also expression levels of certain genes change considerably during the day.

Inter-study comparison and route of administration
Previously, we reported rat liver genes and proteins with altered expression upon intraperitoneal administration of BB [2]. The majority of the genes that changed in the previous study with i.p administration of BB again was identified to change in the present study with oral administration of BB. The overlap between the two, independent, studies demonstrates the robustness of the methods and confirms that our data analysis approach did not allow the introduction of many false positives, frequently raised as a point of concern for cDNA microarray experiments. We conclude that with both routes of administration, i.p. and oral, the same hepatic response was induced at the transcriptome level. The i.p. injection of corn oil induced some subtle effects, while changes induced by the oral administration of corn oil were not readily detected.

Concluding remarks

In summary, gene expression measurements in liver, 6, 24 and 48 h after dosage of several doses of BB yielded a more comprehensive insight into different cellular pathways that are activated when rats are given BB, leading to hepatotoxicity. Results expanded the findings of our earlier experiments [2]. Many changes were in line with the observations from routine toxicological assessments, while also new hypotheses on mechanisms of BB-induced hepatotoxicity were postulated. Recovery of the liver was suggested in response to BB with the altered expression of genes involved in protein synthesis and cytoskeleton rearrangement. After 48 h, the rats in the mid dose group showed no signs of toxicity, concurrent with a gene expression patterns that largely resembled the controls. We identified genes responding to dose levels below 5.0 mmol/kg BW, with some genes responding to oral administration of as low as 0.5 mmol/kg. Thus, we were able to detect significant effects at 2 to 10-fold lower doses with transcriptomics compared to clinical chemistry or histopathology. Genes that could serve as early biomarkers of hepatotoxicity at lower BB exposure were revealed, such as Ho-1, Ephx1, Afar, Mt1. A sample of results from the cDNA microarrays were confirmed by the bDNA assay. Future research will have to establish further whether the changes in gene expression are adverse or protective, and whether they are reversible or irreversible effects.

Acknowledgements

The authors wish to thank Dr. T. van der Lende, E. Wesseling, M. Havekes, R.van de Kerkhof and Dr. F. Schuren for excellent expertise and setting up of the microarray facility. H. Aten and M. van den Wijngaard for assistance in sample isolation.

Chapter IV

Profiles of metabolites and gene expression in rats with chemically induced hepatic necrosis

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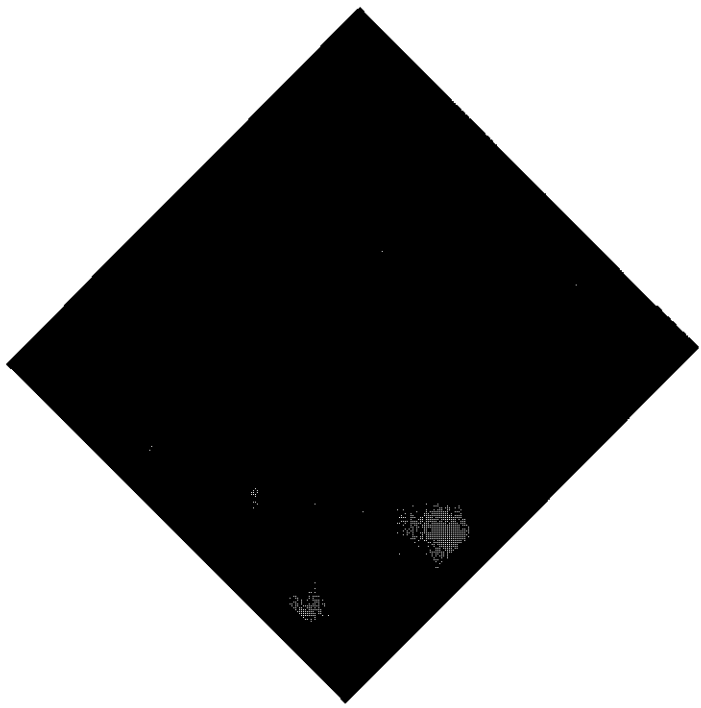

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Abstract

This study analysed changes in gene expression patterns and metabolite levels in plasma or urine in parallel. The aim was to more sensitively detect hepatotoxicity and provide new insights in molecular mechanisms of hepatic necrosis. Rats received the model hepatotoxicant bromobenzene at three dose levels, the highest dose inducing acute centrilobular necrosis. The hepatic transcriptome and plasma and urine metabolite profiles were analysed after 6, 24 and 48 hours, using multivariate statistics.

Principal component analysis showed that molecular profiles from rats with hepatic necrosis differed largely from controls. Changes in levels of genes and metabolites were identified in correlation with the degree of necrosis, providing putative novel markers of hepatotoxicity. Moreover, samples from treated rats were distinguished from controls after exposure to bromobenzene below the concentration that induced hepatotoxicity markers or histopathological changes. Genes with altered expression were involved in oxidative stress, the acute phase response, cytoskeleton structure, apoptosis, biotransformation, glycolysis, cholesterol and fatty acid metabolism. Levels of endogenous metabolites like alanine, lactate, tyrosine and dimethylglycine distinguished plasma from treated and control rats. Complementary, NMR metabolite profiling enabled to distinguish the urine samples based on the exposure levels, primarily through presence of a multitude of bromobenzene-derived metabolites.

Concluding, this parallel analysis of the liver transcriptome and metabolite profiles in plasma enabled to more sensitively detect changes related to hepatotoxicity and discover novel putative markers. Detailed insights in the role of various biological pathways in bromobenzene-induced hepatic necrosis were obtained.

IV.1 Introduction

Previous toxicogenomics studies have shown that both large-scale measurement of gene expression (transcriptomics) and metabolite profiling complement the current methods to identify and discriminate different types of toxicity. Moreover, the new technologies enable to investigate the mechanisms that lead to toxicity. To this date, most studies concentrated on hepatic toxicity. Transcriptomics using DNA-microarrays enabled the discrimination of responses by different classes of hepatotoxicants *in vivo*, as shown by [34],[21],[22]. Hamadeh and co-workers distinguished samples treated with two classes of toxins, and provided more details on the mechanisms of action [27].

In parallel, metabolomics, i.e. metabolite profiling by NMR combined with pattern recognition techniques, has been used to classify urine samples of rats treated with either a liver or a kidney toxicant [31]. Others analysed metabolites in liver, plasma and urine of rats treated with the model hepatotoxicant alpha-naphthylisothiocynanate (ANIT) [33] Urine profiles were analysed in time upon single dosage of ANIT, galactosamine and butylated hydroxytoluene [32]. Time-related differences in metabolite contents were related to the stage of the lesions, and specific changes in metabolite levels were identified for each compound.
While gene expression changes influence biochemical reactions, metabolite levels are determined by those biochemical reactions. Therefore, complementary information is expected from so-called systems toxicology approaches, where transcriptomics, proteomics and/or metabolomics are combined to analyse toxicity in a systematic and holistic manner. Only few experiments integrating results from transcriptomics and metabolite profiling have been described. Very recently, Coen and colleagues reported transcriptomics and metabolomics analyses in mice treated with acetaminophen (paracetamol) [125]. This study demonstrated that analysis of gene expression and metabolite profiles provided complementary insights in APAP-induced hepatic effects. In earlier studies in our laboratory, we evaluated the combined use of transcriptomics and proteomics analyses of hepatotoxicity induced by bromobenzene (BB). Bromobenzene is a well studied model toxicant that causes necrosis in the liver (centrilobular) and kidney. Hepatic biotransformation and toxicity of BB in rat have been reported in detail [74-78]. Because the liver is the target for toxicity induced by many compounds including bulk chemicals, drugs and food ingredients, the characteristics of the response induced by BB could be helpful in understanding hepatotoxicity induced by a variety of xenobiotics.
Transcriptomics and proteomics analyses of hepatotoxicity were evaluated 24 hours after a single *i.p.* dose of BB [2]. A new study was designed to determine the acute hepatotoxic effects at the gene expression level in time, after oral dosage of various concentrations of BB. Hepatic necrosis was observed only at the high dose level after 24 h, though gene expression changes characteristic for BB exposure were observed at 2.5 times lower dose level. A few genes changed at 10 times lower dose levels. expression of several genes was found to change 6 h after dosage. Genes that were statistically significant differentially expressed upon BB dosage were involved in processes like drug metabolism, oxidative stress, GSH synthesis and the acute phase response [3].

Aim of the study

In the present study, the aim was to investigate whether integrated analysis of the data from transcriptomics and metabolite profiling further increased the sensitivity of detection of hepatotoxicity. Our second question was how the combined analysis may expand current

knowledge about the mechanism of chemically-induced hepatotoxicity. Moreover, relationships between gene expression changes and altered metabolite levels were assessed. Thus, NMR-based metabolite profiles of plasma and urine samples, collected from the study described by Heijne and colleagues [3] were combined with the transcriptomics data of this same study. The metabolite profiling aimed at detecting changed concentrations of endogenous metabolites as a result of hepatotoxicity (biomarkers of effect) and of BB-derived metabolites in urine and plasma (biomarkers of exposure). Results from parallel gene expression and metabolite analysis were combined with pre-existing biochemical knowledge in an overall interpretation of the mechanisms of action and effects of BB, a necrosis-inducing chemical, on liver physiology.

IV.2 Materials and methods

Urine and plasma samples were collected from the study by Heijne and colleagues [3], which was also the source of the transcriptomics and toxicity data. Briefly, three doses of bromobenzene (0.5, 2.0 and 5.0 *mmol/kg body weight, dissolved in corn oil, 40% v/v) were administered to male Wistar rats by oral* gavage. Animals were kept under controlled conditions, and the welfare of the animals was maintained in accordance with the general principles governing the use of animals in toxicity experiments of the European Communities (Directive 86/609/EEC) and Dutch legislation (The Experiments on Animals Act, 1997). Nine rats per dose group were treated BB or corn oil, while an additional group was not treated. Three rats per group were sacrificed after 6, 24 and 48 h and blood and livers were collected. Urine was collected for metabolomics between dosing and sacrifice for the 6 h group, and during the last 16 h before sacrifice for the 24 and 48 h groups. During the time urine was collected, rats received water *ad libitum,* but no food.

Transcriptomics
cDNA microarray preparation and hybridization was described previously, [3];[2]. A reference RNA was used, and hybridizations were replicated with swapped fluorophore incorporation (Cy3 and Cy5) in the sample and reference RNA. After quality filtering, lowess normalization and log(base 2) transformation, a set of about 2700 cDNAs was obtained. In present study, we required a correlation higher than 0.6 between the duplicate sets of dye-swap measurements, keeping about 400 genes in the dataset.

NMR analysis
NMR spectra of urine of individual animals were recorded in triplicate, according to (Lamers et al., 2003). Plasma samples were deproteinised by filtration. Filters with a cut-off of 10 kDa (Microcon YM-10, Millipore) were spin-rinsed with 0.5 ml of 0.05 M NaOH followed by 2 × 0.5 ml de-ionised water to avoid contamination of the ultrafiltrate with glycerin. Centrifugation (1h at 10000 rpm) of 0.5 ml plasma over a filter was followed by the centrifugation (1h at 10000 rpm) of 0.5 ml de-ionised water. Filtrates were freeze-dried and reconstituted in 750 µl sodium phosphate buffer (pH 6.0, made up with D_2O) containing 1mM sodium trimethylsilyl-[2,2,3,3,-2H4]-1-propionate (TMSP) as an internal standard. NMR spectra were recorded in a fully automated manner on a Varian UNITY 400 MHz spectrometer (Palo Alto, CA, USA) according to [126].

Data preprocessing and multivariate data analysis
The NMR data file was imported into Winlin (V1.12, TNO, Zeist, The Netherlands). Minor variations from comparable signals in different NMR spectra were adjusted and aligned without loss of resolution. The intensities of signals present in each NMR spectrum were normalised, so that the sum of all intensities was equal to 1. This data set was imported into Matlab (Version 6.5, The MathWorks Inc., Natick, MA, USA) together with the transcriptomics data for preprocessing and multivariate data analysis. The data matrix was centred across time and dose. The sum of squares per variable over time and dose was scaled to 1, and PCA was performed. PCA is a multivariate statistical analysis that reduces the many dimensions of a dataset to few dimensions that describe the majority of the variance. PCA was performed with the PLS toolbox (Version 3.0, Eigenvector Research Inc., Manson, WA, USA), and a score plot visualised differences in gene expression and metabolite profiles. The contribution of each variable to the trend observed in the plot was determined. PCA was also performed on plasma and urine NMR data separately. When score plots revealed differences between groups, the contributions of the original NMR signals to these difference between treated and control were displayed in a factor spectrum. Metabolites were identified using an in-house reference database, and chemical shifts of characteristic metabolites in the NMR spectra are listed in table IV.3.

IV.3 Results

Rats were exposed to the chemical compound bromobenzene and developed hepatic necrosis 24 h after dosing with the high concentration. In parallel, hepatic gene transcription and profiles of plasma and urine metabolites were analysed.

Toxicological examinations

No macroscopic aberrancies of the liver or other organs were observed in any of the rats sacrificed 6 hours after dosage. Histopathology of liver tissue showed no abnormalities in the controls and low dose rats. In some livers a slight presence of mononuclear cell aggregates and/or necrotic hepatocytes was observed. Only in rats that received high concentration of BB, livers had a patchy appearance and gross lesions after 24 hours and focal discoloration after 48 hours. Centrilobular necrosis was found in livers of all those rats, with inter-individual variation in the degree of response (table IV.1). Plasma levels of ASAT, ALAT and bilirubin were markedly elevated, also with inter-individual variation. To correlate the conventional markers of hepatotoxicity with the degree of necrosis in the individual rats, a semi-quantitative score was defined for the hepatocellular necrosis ranging from 0 (no effects) to 10 (very severe centrilobular necrosis) (table IV.1). This score was also used to correlate gene expression levels to necrosis. Figure IV.1 depicts the correlation of ASAT, ALAT, bilirubin, and the relative liver weight with the observed degree of hepatocellular damage.

Apart from the signs of hepatotoxicity, BB significantly decreased plasma levels of glucose at the mid and high dose, after 24 and 48 hours. Cholesterol (n.s.) and phospholipids levels increased by high BB treatment at all time points. Hepatic GSH levels, which play a pivotal role in the hepatotoxicity induced by BB, were slightly decreased six hours after administration of BB. The mid and high dose depleted GSH levels to ~25% of control levels. After 24 hours, GSH levels were nearly restored.

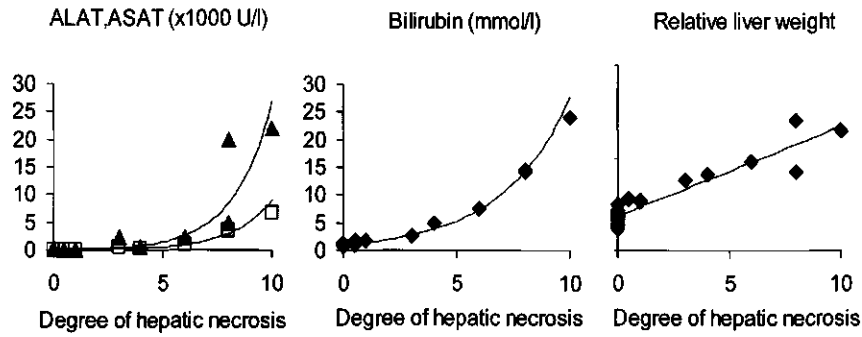

Figure IV.1. *Relation of conventional markers to hepatotoxicity*
Correlation of toxicity markers ALAT, ASAT, bilirubin and relative liver weight with the observed degree of hepatic necrosis in individual rats.

Table IV.1. *Histopathological and clinical chemistry*
Histopathological and clinical chemistry findings in rats, 24 and 48 hours after exposure to mid and high dose of bromobenzene. The degree of hepatic necrosis was expressed with a score (S) between 0-10.

Dose mmol / kg BW	T h	Rat	Rel. liver % of CO	BW g	Gross pathology of the liver	Liver histopathology	S
2.0	**24**	**Mean**	**109%**	**187**	**No gross lesions**	**(Very) Slight mononuclear cell aggregates/ necrotic hepatocytes**	
		62	108%	188	-	Slight mononuclear cell aggregates/ necrotic hepatocytes	1
		64	108%	178	-	Slight mononuclear cell aggregates/ necrotic hepatocytes	1
		66	110%	194	-	Very slight mononuclear cell aggregates/ necrotic hepatocytes	0.5
2.0	**48**	**Mean**	**108%**	**184**	**No gross lesions**	**Very slight mononuclear cell aggregates/ necrotic hepatocytes (1/3)**	
		68	114%	191	-	Very slight mononuclear cell aggregates/ necrotic hepatocytes	0.5
		70	105%	186	-	No abnormalities	0
		72	104%	175	-	No abnormalities	0
5.0	**24**	**Mean**	**131%**	**177**	**Patchy appearance**	**Centrilobular necrosis Nucleolar enlargement (2/3)**	
		80	120%	183	Patchy appearance	Very slight centrilobular necrosis Nucleolar enlargement	3
		82	125%	173	Patchy appearance	Severe centrilobular necrosis Nucleolar enlargement	8
		84	149%	174	Patchy appearance	Very severe centrilobular necrosis	10
5.0	**48**	**Mean**	**142%**	**181**	**Pale appearance others (2/3)**	**Centrilobular necrosis Slight centrilobular fatty change (2/3) Mitotic increase (2/3) Nucleolar enlargement**	
		86	129%	187	No gross lesions	Slight centrilobular necrosis Nucleolar enlargement	4
		88	136%	186	Pale appearance Pronounced lobular pattern	Moderate centrilobular necrosis Slight centrilobular fatty change Slight mitotic increase Nucleolar enlargement	6
		90	161%	170	Pale appearance Red areas Firm tissue	Severe centrilobular necrosis Slight centrilobular fatty change Slight mitotic increase Nucleolar enlargement	8

Transcriptomics analysis and parallel metabolite profiling

BB elicited specific changes in gene expression of many rat liver genes, as reported before [3]. In this study, the profiles of the transcriptomics measurements were combined with the profiles obtained by NMR, describing the metabolite contents of plasma. Consensus PCA [127] was performed using both types of data in one integrated analysis, and results are shown in figure IV.2. This plot indicates that the samples from the high and mid dose groups, collected after 24 and 48 hours were distant from the others, having lower PC1 scores. Most distinct from all the other samples were the samples from rats #84, #82, and #90, that received a high dose of BB. Microscopic examination revealed (very) severe hepatic centrilobular necrosis in those rats. Profiles of rats #80, #86 and #88 were less distant from the controls. Correspondingly, moderate centrilobular necrosis was observed in rat #88, and (very) slight necrosis in rats #86 and #80. The profiles of the rats treated with a mid dose of BB were distinct from the controls after 24 hours. Routine markers were not able to indicate hepatotoxicity in those rats. After 48 hours, rats treated with the mid dose were not distinct from controls. Samples from rats treated with the low dose of BB could not readily be separated from the controls, after 24 or 48 hours. All samples collected after 6 hours were distinct from the other time points in the down right corner of the plot. Treatment with BB resulted in patterns distinct from the controls.

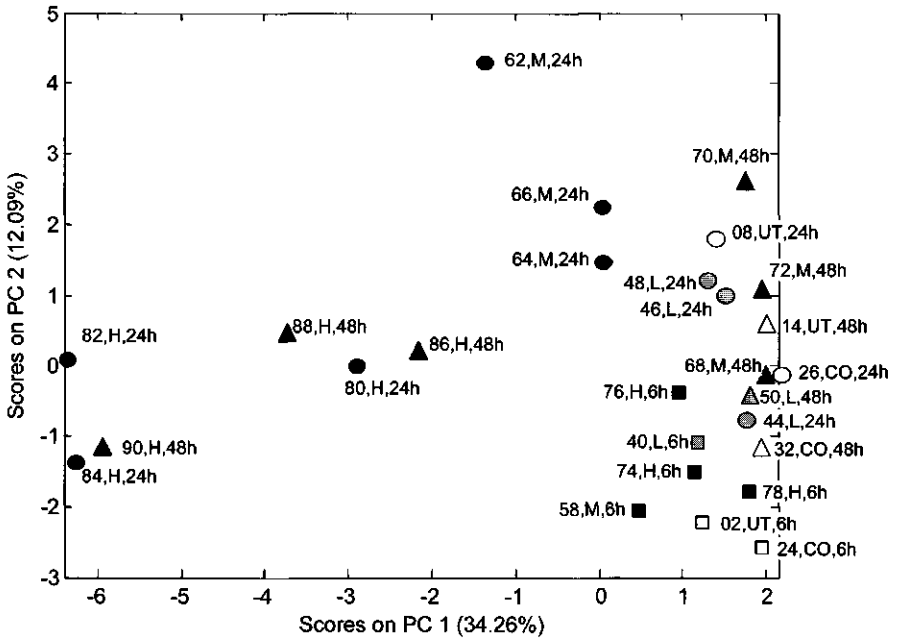

Figure IV.2 *Score plot of consensus PCA*
Consensus PCA was performed using both hepatic transcriptomics and plasma metabolite profiling data in one integrated analysis. The percentage of the total variance explained by the individual PCs is indicated in the plots. Time points: Boxes: 6 h samples, circles: 24 h samples; triangles: 48 h samples. Dose levels: white: controls, light grey: low BB; dark grey: mid BB; black: high BB

Genes and metabolites were sorted according to their contribution to the observed trend, reflecting the degree of hepatic necrosis. Tables IV. 2 A and B list the genes and metabolites with the highest and lowest scores, that therefore putatively correlate with the degree of hepatotoxicity. The levels of gene expression and metabolites listed in are present at either high or low levels in correlation with the necrosis. Many genes with a significant contribution to pattern differences in the PCA were identified to be up- or down regulated by BB with high significance in univariate statistical tests, and the rationale of these changes in terms of toxicology was discussed before [3].

Genes with high scores in the parallel analysis include structure and cytoskeleton-related genes (beta actin, weakly similar to pervin, tubulin), many ribosomal subunits, and other factors involved in protein synthesis (eg. nucleophosmin). Also oxidative stress induced genes (Ho-1, Timp1, peroxiredoxin1, ferritins), hepatic acute phase response genes (orosomucoid 1, fibrinogen gamma) and enzymes involved in glucose metabolism (Gapdh, phosphoglycerate mutase 1, aldolase A) have high rankings. Drug metabolising enzymes like Ephx1, Afar, Gsta and aldo-keto reductases, likely involved in the hepatic biotransformation of bromobenzene, appeared in the upper part of the ranking. Several cell cycle and apoptosis related genes (Bcl2-related protein A1, Pcna, p53, p21 (Waf), EST, highly similar to p53-regulated PA29-T2, cyclin G1) were co-ordinately upregulated. High ranked genes with others functions include casein kinase II, VL30 element and RAN. Plasma metabolites with a high score in the analysis include acetate, choline, phenylalanine and some uncharacterised metabolites.

Genes with low scores include hepatic acute phase response genes like alpha-1-inhibitor, serine protease inhibitor, fibrinogen beta, complement components, drug metabolising enzymes like Cyps, aldehyde dehydrogenases, Fmo3, enzymes involved in fatty acid and cholesterol metabolism (HMG-CoA synthase, Lcat, Star, fatty acid CoA ligase, acyl CoA dehydrogenases) and glucose metabolism (G6pt1, alanine-glyoxylate aminotransferase) Many genes with other functions, like asialoglycoprotein receptor 2, Cathepsin S, and dimethylglycine dehydrogenase had a low score, indicating that they were down regulated compared to the controls. Plasma metabolites with a low score in the analysis include dimethylglycine, tyrosine and glucose.

Table IV.2a: *Genes from consensus principal component analysis*

Highest and lowest ranked genes from consensus principal component analysis. Rank, gene name and Genbank accession number, category, and the correlation to the degree of hepatic necrosis are indicated. Expression of high ranked genes is upregulated, while low ranked genes are downregulated in the samples with a high degree of hepatic necrosis.

Rank	Gb Acc.	Category	Gene name	Correl
1	AA859846	Structure	actin, beta	0.881
2	AA964725	Structure	Weak sim to pervin	0.842
3	AA964496	Structure	High sim to S11222 actin gamma, cytoskeletal	0.853
4	AA957078	Structure	alpha-tubulin	0.842
5	AA924111	glycolysis	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	0.811
6	AI029162	APR	Orosomucoid 1	0.885
8	AA997175	Signal transd.	casein kinase II beta subunit	0.835
10	AA900726	Signal transd.	GTP-binding protein (ral A)	0.789
552	AI070895	Fatty acid	Weakly sim. to acyl-CoA dehydrog., epoxide hydrolase [C.el]	-0.735
553	AA866389	other	lumican	-0.865
554	AA964340	other	syndecan 2	-0.747
555	AA955402	Cysteine	S-adenosylhomocysteine hydrolase	-0.776
556	AA925933	Proteolysis	cathepsin S	-0.801
557	AA819756	Drug metab	arachidonic acid epoxygenase;Cyp2C23	-0.888
558	AI136048	Cholesterol	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2	-0.821
559	AI071033	acute phase	Fibrinogen, B beta polypeptide	-0.820
560	AA997322	Cholesterol	Lecithin-cholesterol acyltransferase (Lcat)	-0.745
561	AA997920	Signal transd.	asialoglycoprotein receptor 2	-0.900

Table IV. 2b. *Metabolites from consensus principal component analysis*

Highest and lowest ranked metabolites from consensus principal component analysis. The rank and chemical shift in the NMR analysis of the (putatively) identified metabolites is indicated. High ranked metabolites are more abundant in treated compared to control plasma samples, and metabolites with a low rank are more abundant in controls.

High rank			Low rank		
Rank	**Shift**	**Metabolite**	**Rank**	**Shift**	**Metabolite**
7	1.475	alanine	430	2.935	dimethylglycine?
9	1.4925	alanine	391	6.91	tyrosine
51	8.4575	formate	390	6.89	tyrosine
61	7.83	unidentified metabolite	389	7.185	tyrosine
70	1.9275	acetate	383	7.2075	tyrosine
73	3.2075	choline?	379	3.4925	glucose
82	3.0075	unidentified metabolite	378	3.245	glucose
100	7.4325	phenylalanine?	376	3.3775	glucose
109	3.5975	choline?	375	3.7325	glucose
110	5.3875	unsaturated lipid?	374	3.4225	glucose
111	3.0275	cysteine?	373	4.64	glucose
114	7.8475	histidine?	372	3.715	glucose
119	7.4125	phenylalanine?	369	5.23	glucose
124	7.3375	phenylalanine?	368	3.09	unidentified metabolite
126	7.375	phenylalanine?	367	3.7475	unidentified metabolite
129	1.005	isoleucine?	366	3.2225	unidentified metabolite
130	3.0525	unidentified metabolite	365	3.4375	unidentified metabolite

Table IV.3. *Chemical shifts of characteristic metabolites in NMR spectra*

Metabolite	**Chemical shift (ppm)**
Diverse BB-metabolites	4.27-4.40 and 5.95-6.35 (region)
Lipids	0.84-1.32 (region)
Lactate	1.33 doublet
Alanine	1.48 doublet
Glutathione (and conjugates)	2.8-3.1 (region)
Cysteine (and conjugates)	3.02 multiplet
Methionine	3.80 triplet
Dimethylglycine	2.74 singlet and 2.94 singlet
Taurine	3.25 triplet and 3.42 triplet
Creatine (and creatinine)	3.04 singlet and 3.93 singlet
Glucose	4.64 doublet, 5.25 doublet and 3.7-3.9 (region)
Tyrosine	6.91 doublet and 7.2 doublet
Nicotinamide/nicotinate	above 8
Formate	8.46 singlet
Choline	3.21 singlet, 3.52 multiplet and 4.07 multiplet

Gene expression markers

The correlation between the level of gene expression and the degree of necrosis in the individual rats was calculated (Table IV.2). Figure IV.3 illustrates expression of ESTs highly similar to actin and pervin, and orosomucoid 1 in relation to hepatic necrosis. Expression levels of asialoglycoprotein receptor 2 and lecithin-cholesterol acyltransferase (Lcat) decreased in concordance with the degree of hepatic damage. In total, 14 genes were found with a positive correlation between 0.80 and 0.89, the highest coefficient. The correlation of the average expression level of these 14 genes with necrosis was 0.969. In parallel, 20 negatively correlated genes were found with an individual correlation to necrosis varying from -0.80 to -0.90. The correlation of the average gene expression of these 20 genes with necrosis was -0.959. This suggests that valuable markers of hepatocellular necrosis consist of combination of gene expressions.

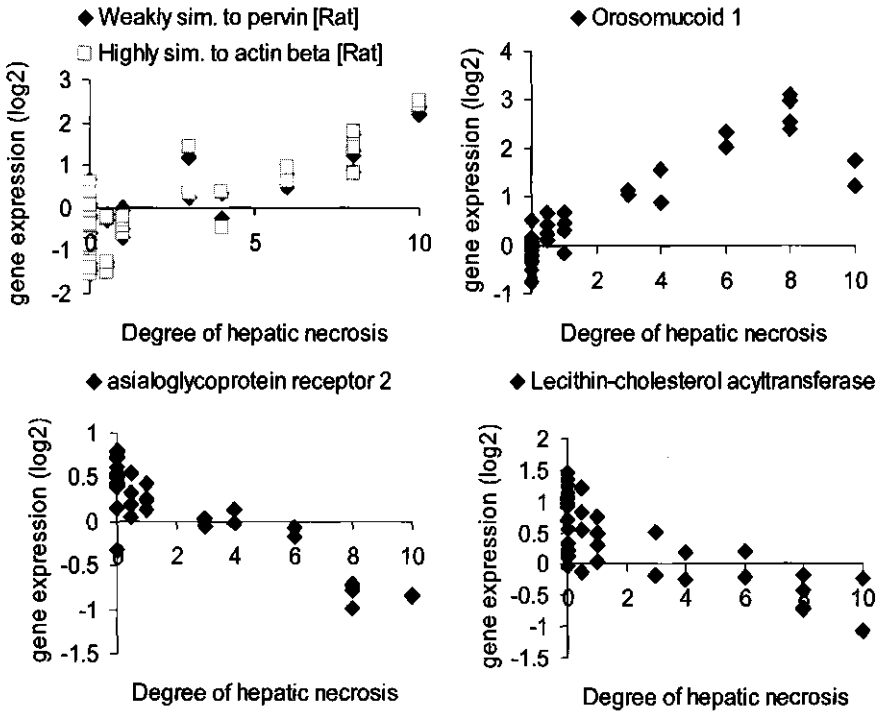

Figure IV.3 *Putative gene expression markers for hepatic necrosis*
Levels of expression of genes that correlate with the observed degree of hepatic necrosis in individual rats.

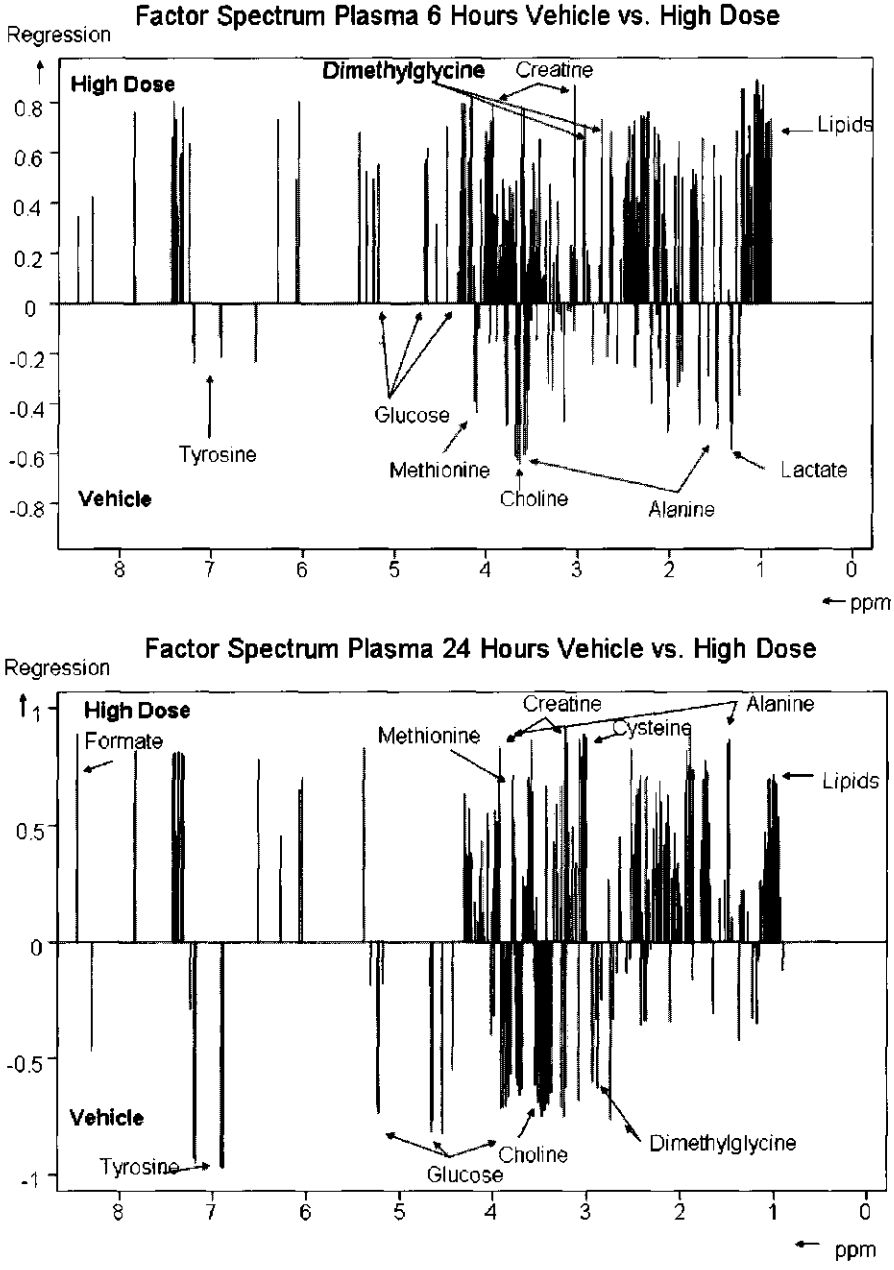

Figure IV. *Plasma metabolite profiling*
Factor Spectra of NMR measurements after principal component analysis.
Upper panel: plasma after 6 hours, Lower panel: plasma after 24 hours

Time and dose-dependent changes in plasma metabolites

Besides the parallel analysis of transcriptomics and metabolite profiling, the plasma NMR data were analysed separately by PCA, and time and dose specific changes in metabolite levels between treated and control samples were visualised in factor spectra. (figure IV.4 a,b). After both high and mid dose of BB, lipid levels were higher than in controls. Clinical chemistry indicated an increase in plasma phospholipids levels upon high but not mid dose treatment. The levels of glucose were higher 6 hours after a high dose of BB, but lower after 24 and 48 hours. These observations were identical to the clinical chemistry measurements. NMR of plasma showed higher levels of creatine and/or creatinine in BB-treated rats, though clinical chemistry did not reveal significant changes in creatinine. The levels of tyrosine were lower 6 and 24 hours after BB, while higher after 48 hours. Methionine, alanine and lactate levels in plasma of BB-treated rats were lower 6 hours after dosage but higher 24 and 48 hours after dosage. Dimethylglycine and taurin levels were increased compared to controls 6 hours after the BB treatment, and decreased after 24 hours. Choline levels were decreased after treatment to mid or high dose of BB.

Profiles of urine metabolites

Also in urine, metabolite NMR profiles were discerned using PCA. Analysis per time point showed that BB treatment changed urine profiles (data not shown). All rat urines collected during the first 6 hours could be distinguished by levels of exposure. By 48 hours after dosage, rats treated with the high concentration of BB could still be recognised from controls by their urine profiles. In order to determine the NMR signals that most significantly differed between the high dose and control group, factor spectra were constructed. Figure IV.5 shows the factor spectrum for rat urine collected during the 6 hours after dosage. Using reference databases, the identity of several peaks was established. Factor spectra revealed the marked presence of BB-derived metabolites like bromphenols, bromcatechols, and quinones in urine. It was not possible to discriminate and identify these various metabolites. Markedly elevated levels of mercapturic acids, derived from GSH-conjugates, were observed after treatment. Methionine levels in urine were higher in the treated rats compared to controls. Formate levels increased after 24 hours in the treated rats, and elevated levels were observed of urocanate and (methyl)histidine, as well as decreased levels of nicotinate, hippurate, phenylalanine/tyrosine and glucose/fructose.

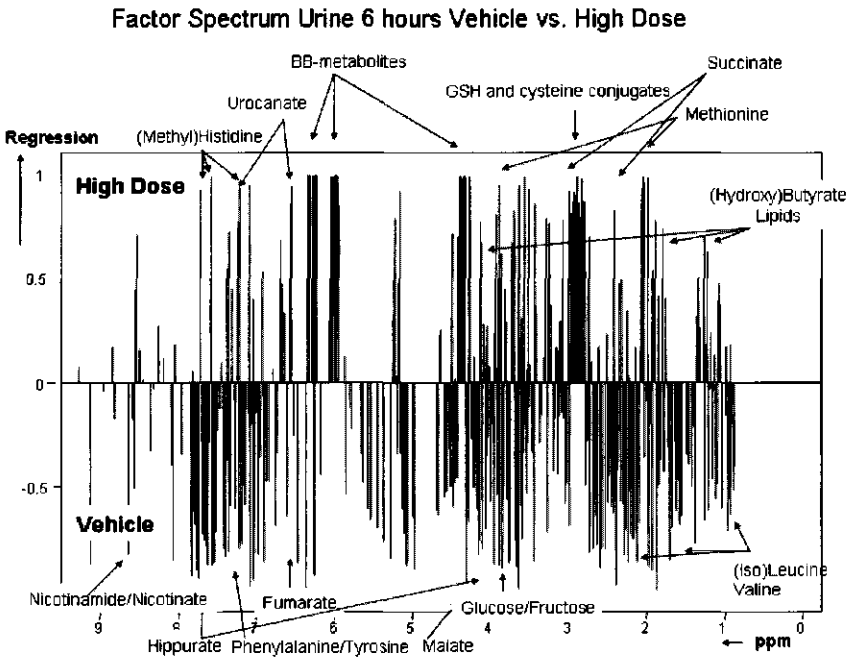

Figure IV.5 *Urine metabolite profiling*
Factor Spectra after principal component of NMR analysis of urine, 6 hours after dosage with bromobenzene, compared to vehicle control.

IV.4 Discussion

This study presents one of the first integrated toxicogenomics studies, where acute hepatotoxicity was analysed at the transcriptome and metabolite level in a time- and dose-dependent manner. An integration of the (raw) datasets of the transcriptomics and metabolomics experiments could increase the sensitivity of detection of hepatotoxicity. Moreover, this could enhance the assessment of relationships between gene expression and metabolite level changes.
When rats were treated with BB, hepatic centrilobular necrosis was observed after 24 hat the high, but not at lower doses. The inter-individual response varied from very slight to very severe hepatic centrilobular necrosis. Individual plasma ALAT, ASAT and bilirubin levels and the increase in relative liver weight correlated with the severity of the necrosis. Complementary to these toxicological observations, the molecular profiles of hepatic gene expression and plasma metabolites were analysed in parallel. Differences between molecular profiles were dependent on the dose and time after dosage. Profiles from the 6 hours time point were distinguishable from other time points. BB treatment at the high dose resulted in highly distinct profiles, while the mid dose altered the profiles up to 24 hours after dosage. At this dose level, conventional signs of hepatotoxicity were not observed. Combining transcriptomics and metabolite profiling did not allow to discriminate samples treated with the low dose from controls.

Markers of gene expression
Gene expression changes were identified in correlation with the degree of hepatic necrosis, providing comprehensive means to diagnose the degree of necrosis. Moreover, if these markers prove to be predictive at earlier time points or lower dose levels, they will improve detection of hepatotoxicity. Changes in these marker gene expression levels could be explained from a mechanistic point of view. The upregulation of cytoskeleton constituents (actin and pervin ao.) with the degree of necrosis indicates remodelling of the cytoskeleton. Presumably, necrosis and repair occur simultaneously in different liver cells, but our experiments using whole liver do not allow to localise the events. The negative correlation of genes like alpha-1-inhibitor and serine protease inhibitor is probably related to the acute phase response, involving altered hepatic synthesis of proteins. When expression levels were averaged for sets of genes, the correlation with the degree of necrosis increased. A further suggestion would be to construct a model of combined sets of positively and negatively correlated genes and metabolites to further increase the relation with hepatocellular necrosis.

Metabolite profiles
Xenobiotic compounds like BB are degraded into many metabolites, and ultimately excreted in urine. BB-derived metabolites could be suitable to monitor exposure and to elucidate routes of biotransformation. Levels of endogenous metabolites that changed after treatment form putative biomarkers of toxicity, and could help to identify the mechanism of hepatotoxicity.
Urine collected from rats exposed to different doses of BB varied in metabolite contents, in agreement with the levels of exposure. Especially shortly after dosage, many water-soluble BB-metabolites were found, like bromphenols, -catechols and -quinones, and mercapturic acids. The lack of reference spectra and insufficient resolution of the separation frustrated

the identification of all corresponding metabolites. Peaks around 6 p.p.m. in the spectra could result from bromphenols, bromcatechols and/or BB-dihydrodiols. Therefore, the precise biotransformation of BB could not be determined. Further efforts to elucidate this based on urine metabolite profiles require techniques like liquid chromatography and mass spectrometry (LC-MS) for identification of the compounds. Few endogenous metabolites, putative markers of hepatotoxicity, were discovered in urine. Levels of methionine were higher 24 hours after BB dosage. Urocanate, related to histidine metabolism, and histidine itself displayed elevated levels. Notably, elevated urocanate levels were also found with galactosamine-induced hepatotoxicity [32].

Contrary to urine, in plasma, distinct signals of BB-derived metabolites were not found. On the other hand, endogenous metabolites in plasma, or combinations of them, could be effective biomarkers of toxicity. Decreased glucose and increased lipid levels measured by NMR were corroborated by clinical chemistry. The levels of formate in plasma, and urine, were increased after 24 and 48 hours. Formate could be produced from dimethylglycine through sarcosine and formaldehyde. Formate is also a product of oxalate in the glyoxylate catabolism, and possibly related to folate synthesis in the one-carbon metabolism.

Biochemical pathways

The most significant effects determined in the parallel analysis of transcriptomics and plasma metabolite profiling were categorised according to biochemical pathways. Changes in gene expression in several pathways were described previously [3]. Other changes, eg. in apoptosis and cell cycle were not noted before. Pathways like glycolysis, GSH and amino acid metabolism were disturbed both at the gene expression and metabolite level, and are described below. Figure IV.6 presents a proposed schematic overview of changes in GSH and amino acid metabolism, associated to bromobenzene-induced hepatic necrosis.

Glycolysis

Glucose levels in plasma decreased in time after BB treatment. This could be ascribed to increased glycolysis, in order to increase the production of energy to restore homeostasis after the toxic insult. Decreasing glucose levels are corresponding with increasing plasma levels of alanine and lactate, products that may be formed by breakdown of glucose when the oxidation of pyruvate is incomplete. Expression of many genes involved in glycolysis, gluconeogenesis and glucose transport was altered. Expression of a glucose transport protein was decreased by BB. From the changes, we conclude that glycolysis enzymes were induced (GAPDH, aldolase A, pyruvate kinase, G6PD and PGAM), and gluconeogenesis was reduced through down regulation of G-6-phosphatase, transport protein 1 (G6pt1), alanine-glyoxylate aminotransferase and pyruvate carboxylase. It is known that the hepatotoxic effects of high doses of APAP are similar to the effects of bromobenzene. In agreement with our findings, APAP was found to decrease glucose levels and was suggested to induce glycolysis based on gene expression and metabolite profile changes, suggestively as a reaction to decreased ATP availability from beta oxidation of fatty acids [125].

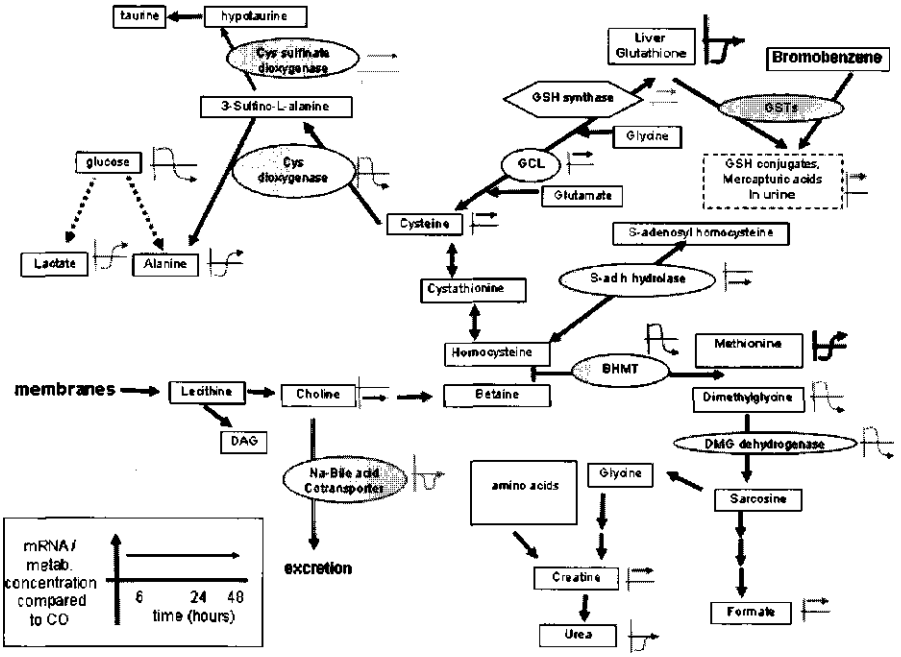

Figure IV.6 *Gene expression and plasma metabolite changes in bromobenzene-induced hepatic necrosis*
Simplified, schematic representation of gene expression and plasma metabolite changes in bromobenzene-induced hepatic necrosis, related to GSH and amino acid metabolism. Ovals represent genes, boxes represent metabolites in plasma. When measured, changes in gene expression or plasma metabolite levels are indicated schematically to the right of each object.

GSH and amino acid metabolism

A central process in the chemically-induced hepatic necrosis is the depletion of GSH levels, which normally protect cells by scavenging of hazardous, reactive molecules. GSH levels decreased to around 25% of controls, 6 hours after oral BB dosage, [3], while total depletion of hepatic GSH was observed 24 h after i.p. administration of BB [2]. GSH is used in conjugation reactions to BB-derived metabolites, catalysed by GSTs. The reduction of GSH levels was accompanied by a decrease of plasma methionine, according to NMR measurements. The GSH depletion was countered through induction of GSH synthase protein [2] and Gclc gene expression [3]. Along with the changes in GSH and methionine levels, related enzymes and metabolites were found to change. GSH and methionine levels are connected via cysteine and homocysteine levels, involving enzyme activity of BHMT. Gene expression of BHMT was found to initially increase, and later decrease upon BB treatment. The expression of S-adenosyl homocysteine hydrolase was decreased. Plasma levels of dimethylglycine, produced in the reaction catalysed by BHMT were found to correlate with the BHMT mRNA levels in time, and also the hepatic dimethylglycine dehydrogenase gene expression levels followed this pattern. Dimethylglycine can be catalysed in a multi-step reaction to formate, which levels were increased both in plasma and

urine after treatment. Induced levels of cysteine in plasma were observed after BB treatment, along with increased gene expression of cysteine dioxygenase, while increased levels of cysteine sulfinic acid decarboxylase were observed before [2]. Plasma tyrosine levels show a characteristic pattern, decreasing drastically 24 hours after high BB, while 48 hours after high BB, levels were highly increased compared to controls. Protein levels of HPD, an enzyme involved in tyrosine metabolism, were found to decrease 24 hours after BB [2]. The level of phenylalanine is related to tyrosine and seems to decrease in plasma due to the treatment.

Conclusion

In summary, this study presents one of the first integrated analyses of transcriptomics and metabolite profiling, revealing additional information in the process of chemically-induced hepatic necrosis. A full merge between the methods awaits technical optimization, especially for the identification of metabolites. Nevertheless, corroborating findings from liver transcriptomics and plasma metabolite profiling aided in the generation of new hypotheses concerning cellular mechanisms putatively related to necrosis, such as changes in cytoskeleton remodelling and acute phase response, apoptosis, glycolysis, amino acid, fatty acid and cholesterol metabolism. Through integration of the datasets, changes were observed before histopathology or clinical chemistry indicated necrosis. Both liver gene and plasma metabolite markers were discovered in correlation with the degree of hepatocellular necrosis in individual animals. Through measurement of urine metabolite profiles, exposure was rapidly recognised.

Acknowledgements

The authors thank dr. T. van der Lende, E. Wesseling, M. Havekes, R. van de Kerkhof and dr. F. Schuren for excellent expertise and setting up of the microarray facility. M. van den Wijngaard for assistance in sample isolation. We gratefully thank dr. A. Smilde for helpful discussions on multivariate statistics.

Chapter V

Liver gene expression profiles in relation to subacute toxicity in rats exposed to benzene

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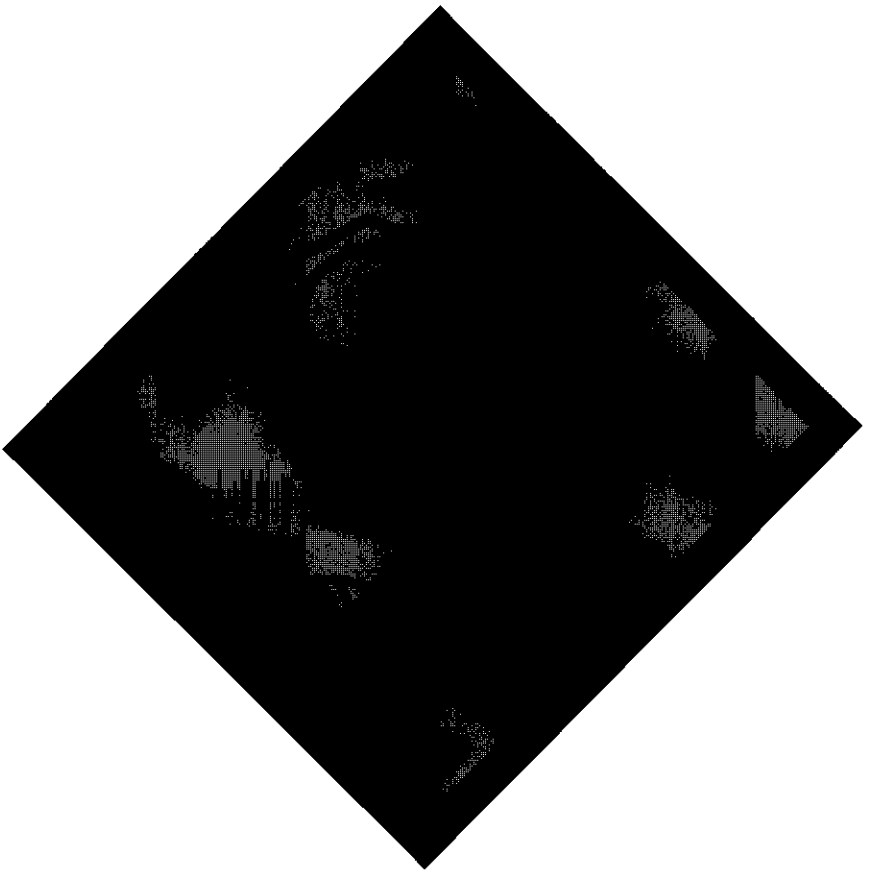

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Abstract

The number of published toxicogenomics studies is rapidly increasing. Most of the studies so far have analysed hepatic gene expression changes upon exposure to well-known hepatotoxic compounds. To delineate the specificity of gene expression changes induced by (potentially) hepatotoxic compounds, the present study analysed the effects of the industrial chemical benzene, also found in automobile exhaust and cigarette smoke. Benzene is not typically toxic in liver, and critical organs are bone marrow and blood, after bioactivation in the liver. Benzene increased liver weight and possibly other adverse effects in liver.

This toxicogenomics study assessed hepatic gene expression in relation to subacute toxicity in rats repeatedly exposed to benzene. A metabolomics approach using NMR and pattern recognition was used to assess exposure to benzene through characteristic metabolite profiles in urine.

Oral exposure to 200 and 800 mg/kg/day, but not 10 mg/kg/day, for 28-days, induced hematotoxicity in male F344 rats. It slightly reduced body weights, increased relative liver weights and elevated hepatic glutathione levels, but no histopathological abnormalities were found in liver. Using oligonucleotide microarrays, changes in gene expression were identified, even at exposure to 10 mg/kg/day. A marked dos-dependency in the hepatic effects was not seen, probably because hematotoxicity was the critical effect. Specific gene expression changes were observed, while some effects were also seen with model hepatotoxicants bromobenzene and acetaminophen. Modulated genes were involved in biotransformation, glutathione synthesis, fatty acid and cholesterol metabolism and other pathways. Induction of cytochrome P450 2e1 and epoxide hydrolase suggested enhanced bioactivation, while induction of various (conjugation) enzymes could enhance detoxification. In conclusion, this study identified and characterised hepatic gene expression changes upon repeated exposure to benzene, in relation to the subacute toxicity.

V.I Introduction

Toxicogenomics studies

Gene expression changes in livers of rats exposed to model hepatotoxicants were analysed in detail in several toxicogenomics studies. The effects of enzyme inducers (eg. phenobarbital) and agents that induce peroxisome proliferation (eg. Wy14,643) were distinguished and mechanisms of hepatic toxicity were studied [27], [34],[21],[22]. Our laboratory identified characteristic changes at the gene and protein expression level, concurring with acute hepatic necrosis induced by bromobenzene (BB) [2], [3]. Heinloth and colleagues [69] analysed effects of high doses of acetaminophen (APAP, or paracetamol), which also induced acute hepatic centrilobular necrosis. Changes in gene expression were identified in various biological pathways, such as biotransformation, glycolysis and metabolism of proteins, fatty acids and glutathione. Nevertheless, the relation between hepatotoxicity and (specific) changes in gene expression profiles, is still unclear.

Aim of this study

To shed light on the specificity of gene expression changes in liver, the present study analysed the response to benzene, which is not a typical hepatotoxicant. Benzene is a widely used chemical that is bioactivated in liver and ultimately induces hematotoxicity and myelotoxicity. Benzene induced relative liver weight, and reactive metabolites might induce other adverse effects in liver. Therefore, hepatic gene expression changes in rats exposed to benzene were analysed and compared with effects of model hepatotoxicants.

Benzene toxicity

Benzene is an industrial chemical widely used in the production of paints, resins, rubber, inks and dyes. Common sources of environmental exposure include gasoline fumes, automobile exhaust and cigarette smoke. Benzene is genotoxic in animals and possibly in humans [128,129]. The most important effect of benzene is hematopoietic toxicity, depleting white blood cells [130]. Chronic exposure induced bone marrow toxicity (myelotoxicity) in humans and animals, and increased the risk of acute myelogenous leukaemia [119],[128],[131]. Hydroquinone metabolites of benzene accumulate in the bone marrow. There, myeloperoxidases produce p-benzoquinones, which are protein-reactive and possibly responsible for myelotoxicity by damaging the stem cells and progenitor cells [132-135]. Benzoquinones can be detoxified by Nqo1, though this enzyme is expressed at low levels in the bone marrow, making this tissue especially prone to damage by these metabolites. It was shown that exposure to benzene reduced the number of lymphocytes, and increased the number of granulocytes in the bone marrow [136]. Benzene metabolites formed covalent adducts with proteins (eg. hemoglobin) in human blood [137], and also with nucleic acids in rats [138-140], in correlation with decreased RNA synthesis [141].

Metabolism of benzene

Toxicity of benzene depends on bioactivation, primarily by Cyp2e1 in the liver. Subsequently formed benzene oxide is nonenzymatically rearranged to phenol, catalysed by Ephx1 to dihydrodiol and to catechols [142]. Detoxification involves conjugation by GSTs, UGTs and sulfotransferases. Benzene oxide can also be metabolised to the (hemato)toxic metabolite *t,t*-muconaldehyde [143], and ultimately excreted in urine as *t,t*-muconic acid [130]; [144], [131]; [145] Benene biotransformation was dependent on the dose, and the relative amount of muconic acid and other toxic metabolites decreased as the dose increased (eg. [146]).

Urine metabolomics

Many different metabolites of benzene are ultimately excreted in urine. Therefore, the highly characteristic metabolite profile could be suitable to assess exposure to benzene in urine. A metabolomics approach combining NMR spectroscopy and mathematical pattern recognition was followed, comparable to previous applications of this technology [65] [33],[126],[4].

In summary, the hepatic response to benzene at the gene expression level assessed in relation to conventional toxicological observations, and to gene expression changes found with model hepatotoxicants. This study also investigated whether changes in gene expression could provide insight in the mechanisms of hepatic bioactivation and toxicity of benzene.

V.2 Materials and methods

Benzene (> 99% purity) was obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Corn oil was obtained from Remia (Den Dolder, The Netherlands).
Male F344 rats (CDFF® (F344)/CrlBR) were obtained from Charles River Wiga GmbH (Sulzfeld, Germany). The animals were maintained in accordance with the general principles governing the use of animals in toxicity experiments of the European Communities (Directive 86/609/EEC) and Dutch legislation (The Experiments on Animals Act, 1997). The rats were housed conventionally, in macrolon cages with stainless steel grid covers and wood shavings as bedding material, five rats per cage, in a room maintained at 22 ± 2°C with a relative humidity of 30-70% and a 12-h light/dark cycle. Throughout the study, feed (a powdered, cereal-based, closed-formula rodent diet (Rat and mouse No. 3 Breeding diet; RM3) from Special Diets Services (Witham, England)) and tap water were freely available. Following an acclimatisation period of 8 days, the 5-week old rats were allocated randomly to the groups, in such a way that the mean body weights were about the same in all groups.
Benzene was dissolved in corn oil and administered by oral gavage in a volume of 5 ml per kg body weight at dose levels of 10, 200 and 800 mg/kg/day, once daily, for 28 consecutive days. The last dose was given on the day before scheduled sacrifice. The highest dose level was intended to induce toxic effects but no severe suffering, death or marked growth retardation. The low and mid dose levels were selected to demonstrate dose-effect relationships, and no toxic effects at the lowest dose level, based on routine toxicity studies, reviewed in [128] Controls were dosed with corn oil (5 ml/kg body weight) only. The dose volumes were adjusted twice per week to changes in body weight. Fresh benzene solutions were prepared once per week and stored at 2-10°C in portions sufficient for one day.

Observations and toxicological analyses

General clinical observations were made daily before and after dosing. The rats were weighed twice per week and at final necropsy. Food consumption was measured per cage, over successive periods of 7 days (wk 1-3) or 4 days (wk 4). Water consumption was measured per cage, on three consecutive days in wk 1-3. In addition, water consumption of individual animals was measured during the 24 h urine collection period in wk 4. Neurobehavioural functioning was evaluated on day 22 before the daily dosing. During one minute, the animal was observed in an open arena for gait characteristics, arousal and the occurrence of clonic and/or tonic convulsions. Next, sensorimotor reflex testing (aerial righting, responses to the approach of a pencil, touch of a pencil to the rump, click stimulus and tail pinch), gripstrength (forelimb and hindlimb) measurements and landing foot splay were performed.

Organ weights and pathology

At the end of the treatment period, the rats were killed by exsanguination from the abdominal aorta under CO_2/O_2 anaesthesia and examined grossly for pathological changes. The kidneys, liver, prostate, spleen, testes and thymus were weighed. After fixation in a 4% neutral buffered solution of formaldehyde, the kidneys, liver, spleen and thymus were processed, embedded in paraffin, sectioned at 5 µm, stained with haematoxylin and eosin, and examined by light microscopy. Parts of these organs were snap-frozen in liquid nitrogen and stored at < -70°C.

Urine analysis and metabolomics

Urine was collected towards the end of the fourth week of treatment. The rats were kept in metabolism cages (one rat/cage) for two consecutive days, with food and water freely available. After one day of acclimatisation, 24 h urine samples were collected, in ice-cooled containers. Each sample was characterised with respect to appearance, volume, density (Bellingham and Stanley refractometer), total protein (Cobas-Bio centrifugal analyser), glucose, creatinine, alkaline phosphatase (ALP), gamma glutamyl transferase (GGT), lactate dehydrogenase (LDH) and N-acetyl-ß-glucosaminidase (NAG) (Automatic analyser model 911 from Hitachi, Japan). Protein, glucose, creatinine and the enzyme activities were expressed as units excreted per 24 h by multiplying the concentrations measured by the volume of the urine produced.
The urine samples were also analysed with a metabolomics approach, by NMR spectroscopy according to Lamers *et al.* [126] and Heijne *et al.* [4]. The multivariate statistical technique PCDA was used to determine differences in the NMR profiles of the metabolites in urine. PCDA [147] is a semi-supervised method based on principal component analysis that uses the dose levels as a discriminant to optimise the contrast between the treatment groups. After calculations, the differences between the

individual profiles were visualised in a score plot. Along the axis of the score plot, the D#1 and D#2 values were plotted. The score plot displayed every individual profile (in this case NMR spectrum) as one dot, and the distance between the dots reflects the degree of similarity between the individual profiles.

Haematology

At final necropsy, blood samples were collected from the abdominal aorta using K2-EDTA as anticoagulant. Each sample was analysed for haemoglobin, packed cell volume, red blood cell count (RBC), total white blood cell count (WBC), thrombocyte count (Sysmex K-1000 Haematology Analyser from Toa Medical Electronics Co., Ltd, Japan), differential white blood cell counts (microscopic examination of stained blood smears), and prothrombin time (Normotest, Nyegaard and Co. A/S, Norway). Mean corpuscular volume, mean corpuscular haemoglobin concentration and absolute white blood cell counts were calculated.

Clinical chemistry

At final necropsy, blood samples were collected from the abdominal aorta in heparinised tubes. The samples were centrifuged and the plasma was analysed for aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), ALP, GGT, LDH, total protein, albumin, glucose (non-fasting), urea, creatinine, total bilirubin, total cholesterol, triglycerides and phospholipids (Automatic analyser model 911 from Hitachi, Japan). The albumin/globulin ratio was calculated.

Hepatic GSH and CYP

Frozen liver tissue was homogenised in 0.01 M Tris-HCl/0.14 M KCl, pH 7.4 buffer (1:3 w/w) using a Potter-Elvejhem tissue homogeniser. Post-mitochondrial supernatant (S9-mix) was prepared by centrifugation at 10 000 g for 20 min. An aliquot of the S9-mix was stored at <-20°C until analysed for GSH. Microsomes for Cyp analysis were prepared by ultracentrifugation (100 000 g for 75 min) of S9-mix. The microsomal pellet was resuspended in homogenisation buffer and centrifuged for 60 min at 100 000 g. The washed microsomes were suspended in homogenisation buffer and stored at <-70°C until analysis. The S9-mix and microsomes were analysed for protein content using the Bradford method. Total glutathione (GSH) was measured according to [81] Total hepatic Cyp protein contents were determined according to [148].

Statistical analysis of toxicity parameters

Body weights were evaluated by one-way analysis of covariance (covariate: body weight on day 0) followed by Dunnett's multiple comparison tests. The continuous neurobehavioral parameters (grip strength, landing foot splay), water consumption measured per animal, red blood cell and clotting potential variables, total white blood cell counts, absolute differential white blood cell counts, clinical chemistry values, routine urinary parameters (except for appearance), organ weights and hepatic levels of Cyps and glutathione were evaluated by one-way analysis of variance followed by Dunnett's multiple comparison tests. Rank order neurobehavioral data (gait score, arousal, reflexes), relative differential white blood cell counts, and urinary appearance were evaluated by Kruskal-Wallis non-parametric analysis of variance followed by Mann-Whitney U-tests. Histopathological findings were analysed by Fisher's exact probability test. All analyses were two-sided.

Liver RNA extraction

Frozen liver samples were pulverised with mortar and pestle in liquid nitrogen before isolation of total RNA. Total RNA was extracted using Trizol according to the manufacturer's protocol (Invitrogen, Breda, The Netherlands) Total RNA was further purified using the RNEasy RNA purification kit, including a DNA digestion by RNAse-free DNAseI incubation (Qiagen, Westburg B.V., Leusden, Netherlands). RNA was checked for purity and stability by gel electrophoresis and the concentration was calculated from the OD_{260} as determined spectrophotometrically. The yield of total RNA was calculated per g of frozen, pulverised liver.

DNA microarray-based gene expression measurement (transcriptomics)

A referenced design was used for microarray hybridisation, to account for dye-dependent effects, which are generally recognised to influence the outcome of dual-channel microarray experiments. Tester samples, equal amounts of RNA of each rat liver pooled per dose group, were fluorescently labelled with Cy5, while reference material, obtained from pooled RNA of the livers of the control rats, was labelled with Cy3.

Oligonucleotide microarray preparation

Oligonucleotide microarrays were produced in collaboration with the University Medical Center Utrecht Genomics Laboratory [149]. The method according to [48], was described before, [2]. Briefly, about 5000 different oligonucleotide fragments (Qiagen Operon, Westburg B.V., Leusden, The Netherlands) were arrayed in duplicate on glass slides. Additionally, spots with only buffer and control DNAs were included with no significant homology to rat DNAs.

RNA labeling, hybridisation and scanning

In vitro transcription reactions with indirect fluorescence labelling were performed according to Heijne et al. [2]. Hybridisation was performed for 16 hours at 42°C. Slides were washed and scanned, using a Packard Scanarray confocal laser scanner, using different lasers for the cy3 (wavelength 550nm) and cy5 (wavelength 650nm) channel. Two TIFF images per slide were saved for analysis using Imagene 5.0 (Biodiscovery Inc., El Segundo,CA, USA). Settings were applied to automatically flag weak or negative signals and spots with a non-homogenous signal. Excel (Microsoft Corporation, Redmond, WA,USA) and SAS (SAS, Cary, USA) were used to further process and analyse the data.

Microarray quality criteria

Technical replicate hybridisations of the samples were performed until 4 microarrays were obtained that met our quality criteria. These criteria 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.

Data processing

After image analysis, the local background intensity was subtracted from the signal intensity for each spot. Background intensities outside the cDNA spots were very low and homogeneous. Fluorescence intensity in control spots that account for non-specific hybridisation and background fluorescence were used to determine a minimal signal intensity threshold value of 1.5 for the two channels. Flagged spots and controls were excluded from further interpretation. Genes for which less than 70 % of the microarrays delivered a reliable signal were excluded from further interpretation. Ratios of the background-corrected intensities of tester over reference were calculated for each slide and, to account for technical variations introduced during labeling or hybridisation, data were normalised using the lowess algorithm [103] in SAS. Data were log-transformed, with base 2, and excluded values were replaced by 0. Statistical analysis of the changes was performed using One-way ANOVA in SAS. Genes with an ANOVA p-value < 0.01 were considered to have changed statistically significantly. Student's t-tests were performed to determine at which dose significant changes occurred. For presentation in the tables, the average log(2) gene expression levels were corrected for the expression levels in the controls. The ANOVA was performed with, and repeated without, the three slides identified by PCA as aberrant from the others, and similar results were obtained. PCA and PCDA were performed on the filtered, normalised and log(2) transformed data. Excluded values were replaced by 0.

An unsupervised PCA was used, according to Heijne et al., (2003) [2], to assess the differences between the individual microarray profiles (not shown). This analysis indicated that, though meeting the quality criteria, several microarray slides (s26, s91, s93) were aberrant from the others, independent of the treatment. These slides were excluded in the subsequent PCDA. The PCDA was performed as described above for the urine metabolomics data, and a score plot was drawn. The genes with the largest significance for the differences between samples treated with benzene and controls were found by ranking of the genes according to D#1.

V.3 Results

Rats were exposed to benzene for 28 days and hepatic gene expression was determined, complementary to conventional toxicity parameters and metabolomics analysis of the urine. No chemical-related mortality occurred, and the daily clinical observations and the neurobehavioral assessment showed no treatment-related changes. After dosing, animals given benzene at 800 mg/kg/day showed the following, transient symptoms: (facial) tremors on at least 11 days, walking with hunched back, excited, lethargy, ataxia, blepharospasm, flattened ears, increased respiratory frequency on one or a few days in weeks 1 and 2. Body weight (Table V.1) and food consumption were dose-dependently decreased from 200 mg/kg/day. The terminal body weight was decreased by about 10% at 200 mg/kg/day and about 20% at 800 mg/kg/day. Water consumption was not affected.

Organ weights and pathology

Animals given benzene showed statistically significant increased relative liver weights at 800 mg/kg/day, and decreased weights of spleen and thymus (table V.1). Studies have shown that in subacute studies, an increase in relative liver weight should be considered as a liver-specific change that can not be ascribed to reduction of body weight only [150],[151]. Various mechanisms that could explain the increased relative liver weight were described [151]. In the case of benzene, the increased expression of drug metabolism enzymes in the liver might be the most important reason for the relative increase of the liver weight. Concurring histopathological effects were not observed, though at higher dose levels, induction or rough endoplasmatic reticulum (RER) and hypertrophy might be found. Macroscopic examination revealed no changes which could be ascribed to the administration of benzene. Histopathological examination showed a treatment-related decrease in the number of B-lymphocytes in the marginal zone of the spleen at 800 mg/kg/day (incidences: 0/5 at 0, 10 and 200 mg/kg/day , 4/5 at 800 mg/kg/day).

Table V.1. *Body weights and relative organ weights.*
Values represent group means (n=5) and standard deviations.

Benzene (mg/kg/day)	body (g)	rel. liver (g/kg BW)	rel. kidney (g/kg BW)	rel. spleen (g/kg BW)	rel. thymus (g/kg BW)
0	210.7 ± 7.2	40.1 ± 0.6	7.15 ± 0.07	2.31 ± 0.04	1.80 ± 0.05
10	205.6 ± 4.6	39.7 ± 0.5	7.08 ± 0.03	2.35 ± 0.04	1.74 ± 0.04
200	194.0 ± 7.6	41.1 ± 0.6	7.19 ± 0.09	1.99 ± 0.04**	1.74 ± 0.07
800	169.8 ± 1.7**	44.3 ± 1.7*	7.47 ± 0.14	1.71 ± 0.06**	1.56 ± 0.04*

Statistical analysis: ANOVA + Dunnetts tests: significance *: $p < 0.05$, **: $p < 0.01$

Urine analyses and metabolomics

Conventional urine analysis showed statistically significant decreases in the amount of creatinine excreted per 24 h and in the concentrations of glucose and protein at 800 mg/kg/day (data not shown). The lower concentrations of glucose and protein were not considered to be of toxicological significance because the excretion of these analytes per 24 hr was not altered significantly.

Metabolomics analysis of urine samples of the rats determined the metabolite contents by NMR spectroscopy. PCDA was used to discern the NMR patterns of the samples from the different exposure levels. Results of the PCDA are visualised in figure V.1. This score plot reveals the differences between the urine samples, based on the treatments. The pattern of the metabolite contents in urine changed in a dose-dependent manner. While the low dose exposure samples closely resembled the controls, the high dose samples were very different. The interanimal variation within the groups (triplicate measurements of 5 rat urines) was smaller than the difference between the treatment groups.

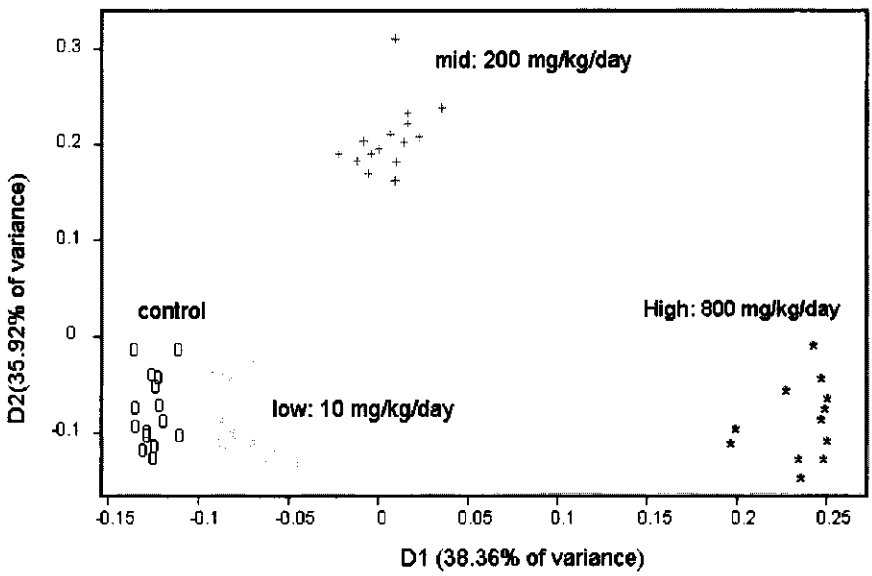

Figure V.1. *Urine metabolomics : PCDA Score plot*

Score plot of principal component analysis with discriminant (PCDA) of NMR spectra, reflecting the metabolite profiles of urine. Each symbol in the score plot represents an individual NMR spectrum. Per group, samples of 5 rats were measured in triplicate. The symbols represent: 0: controls; x: exposure to low dose of benzene (10mg/kg/day); + : mid dose of benzene (200mg/kg/day); and *: high dose of benzene (800 mg/kg/day).

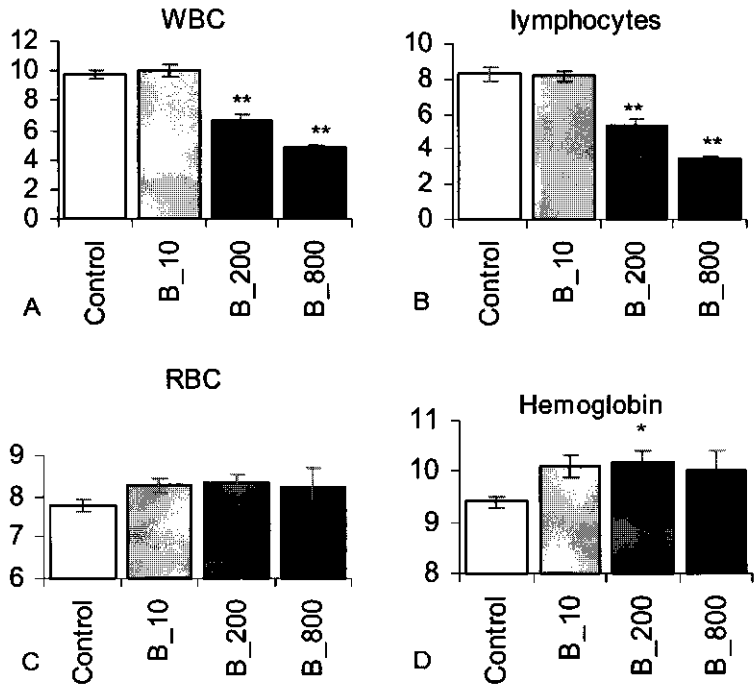

Figure V.2. *Hematology*
Analysis of blood of rats exposed for 28 days to 0, 10, 200 or 800 mg/kg/day of benzene. Group average values and s.e.m. are represented. **panel A.** White blood cell counts (WBC) , **panel B.** Numbers of lymphocytes, **panel C.** Red blood cell counts (RBC), **panel D.** Hemoglobin levels in blood. Statistical significance is denoted with **: $p < 0.01$, *: $p < 0.05$.

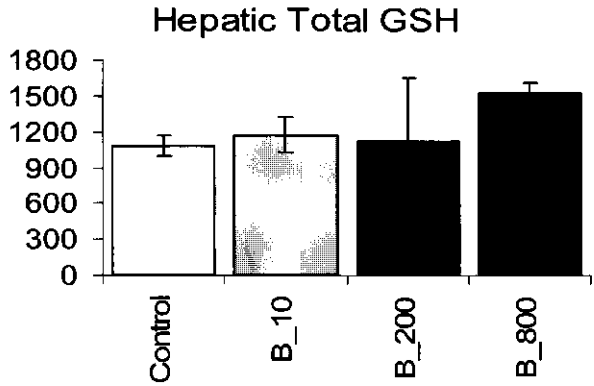

Figure V.3. *Hepatic glutathione (GSH) levels.*
Group average values and standard deviations are represented. Statistical significance ($p < 0.01$) is denoted with **.

Analysis of blood and liver

Haematology revealed that total white blood cell count (Figure V.2A) and lymphocyte count (Figure V.2B) were dose-dependently decreased from 200 mg/kg/day . Red blood cell count and hemoglobin levels slightly increased (n.s.) (Figure V.2C and 2D). Clinical chemistry values showed no statistically significant differences in plasma of animals given benzene and controls (data not shown). The levels of total glutathione in liver were dose-dependently increased from 200 mg/kg/day ($p < 0.01$) (Figure V.3). The total hepatic Cyp contents showed no significant differences between animals given benzene and controls. The yield of the RNA isolation from pulverised and frozen liver tissue was lower in the samples of the benzene-treated rats (data not shown). The yield in the high dose group was 66 % of the yield in the controls (n=5, Student's T-test $p = 0.01$).

Gene expression analysis

Liver gene expression was measured in quadruplo per treatment group. Statistical analysis of variance (ANOVA) was used to identify which genes were significantly altered by benzene at one or more dose levels. Table V.2 lists the genes for which expression was statistically significantly modulated by benzene. Exposure to benzene did not elicit changes in mRNA levels larger than four fold, though many small changes were statistically significant at one or more dose levels. Genes with altered expression upon benzene treatment were categorised according to biological processes including drug metabolism, GSH metabolism and oxidative stress, fatty acid and cholesterol metabolism, glycolysis and protein synthesis. For a selection of relevant genes, the expression levels were shown in figure V.5.

Table V.2 A *Gene expression changes related to biotransformation*
Per gene, the average (n=4, duplicate spots averaged) log(2) fold change in expression relative to the control level is listed for all three dose levels. Levels of significance in the T-tests are indicated next to the fold changes, as follows: *** $p < 0.001$; ** $p < 0.01$; In the last column **(A)**, the level of significance in the ANOVA is provided. The Genbank accession number **(Acc#)** and the chromosomal location **(Chr.)** of the gene in rat are provided.

Gene Name	Acc#	Chr.	10	200	800	A
				mg/kg BW /day		
Retinoid X receptor alpha	NM_012805	3p12	1.53	1.61 ***	1.68 **	*
Aldehyde dehydrogenase 1, subfamily A1	NM_022407	5	-0.20	0.76 ***	1.33 ***	***
UDP-glucuronosyltransferase, Phb-inducible	M13506	14p21	0.19	0.78 **	1.09 **	***
Alcohol dehydrogenase (class I), alpha	NM_019286	2q44	0.30	0.55	1.08 **	***
Aflatoxin B1 aldehyde reductase	NM_013215	5q36	-0.08	0.46	1.06 **	**
Cytochrome P450, 8b1, sterol 12 alpha-hydrolase	NM_031241	8q32	0.30	0.02	0.99 **	**
senescence marker protein 2A, exons 1 and 2	NM_012695	1q21	0.47 **	0.72 **	0.90 **	***
Alcohol dehydrogenase (class I), alpha	M15327	2q44	-0.12	0.20	0.84 *	***
Diaphorase (NADH/NADPH) (NQO1)	NM_017000	19q12	0.44	0.72 **	0.82	
Glutathione-S-transferase, alpha type (Ya)	NM_031509	8q31	0.13	0.32	0.74 ***	***
Cytochrome P450, subfamily 2e1	NM_031543	-	0.29	0.36	0.72 **	*
UDP-glucuronosyltransferase 1 family, member 1	AF461734	9q35	0.49 **	0.51 ***	0.61 *	***
Epoxide hydrolase 1 (microsomal)	NM_012844	13q26	0.02	0.43	0.57 *	**
Sulfotransferase hydroxysteroid 2	M31363	1q21-q22	0.10	0.50 ***	0.53 **	***
Cytochrome P450 15-beta	NM_031572	1q53	-0.05	-0.25	-0.67 **	**

Table V.2 B *Gene expression changes, related to fatty acids, lipids and sterols metabolism*
Per gene, the average (n=4, duplicate spots averaged) log(2) fold change in expression relative to the control level is listed for all three dose levels. Levels of significance in the T-tests are indicated next to the fold changes, as follows: *** $p < 0.001$; ** $p < 0.01$; In the last column **(A)**, the level of significance in the ANOVA is provided. The Genbank accession number **(Acc#)** and the chromosomal location **(Chr.)** of the gene in rat are provided.

Gene Name	Acc#	Chr.	10	200	800	A
			mg/kg BW /day			
Cd36 antigen	NM_031561	?	0.31	0.40	1.18 **	**
apolipoprotein B (apoB)	X55969	6q14	2.07 **	1.71 ***	1.03	**
Farensyl diphosphate synthase	NM_031840	2q34	-0.15	-0.13	0.92 **	**
3-hydroxy-3-methylglutaryl-Coenzyme A reductase	NM_013134	2q12	0.15	0.51	0.80	**
7-dehydrocholesterol reductase	NM_022389	1q41	-0.05	0.02	0.73 *	**
Squalene epoxidase	NM_017136	7q33	-0.14	0.15	0.72 ***	***
Sterol response element binding factor SREBf1;strain BN-Lx/Cub ADD1/SREBP-1c	AF286470	10q22	0.53	0.24	0.70 **	*
Isopentenyl-diphosphate delta isomerase	AF003835	17q12.1	0.08	0.42	0.67 **	**
Fatty acid desaturase 1	AF320509	1q43	-0.23	0.12	0.49 *	***
acetyl-coenzyme A carboxylase	X53003	10q26	-0.31 *	-0.25	0.43	**
Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase	NM_012584	2q34	0.18	0.21 *	0.41 *	**
Phospholipase A2, group IVA (cytos, Ca-dependent)	U38376	13q21	-0.47 *	-0.14	-0.40	**
Liver-specific bHLH-Zip transcription factor 7	NM_032616	1q21	-0.40	-0.55 ***	-0.29	
Rab geranylgeranyl transferase component, beta	L10416	2q45	-0.42 **	-0.40 **	-0.29	**
2-hydroxyphytanoyl-CoA lyase	AJ245707	16p16	-0.54 **	-0.76 **	-0.16	***

Table V. 2C *Gene expression changes related to other processes*

Genes with most pronounced response to benzene are shown. Per gene, the average (n=4,and duplicate spots) log(2) fold change in expression relative to the controls is listed for all three dose levels. '
Levels of significance in the T-tests and ANOVA (**A**) are indicated next to the fold changes, as follows:
*** p < 0.001; ** p < 0.01; *: p<0.05
The Genbank accession number (**Acc#**) and the chromosomal location (**Chr.**) of the gene in rat are provided.

Gene Name	Acc#	Chr.	10	200	800	A
			mg/kg BW /day			
Actin, beta	NM_031144	12p11	0.58 **	0.58 **	0.47	**
Activating transcription factor ATF-4	NM_024403	7q34	-0.67 ***	-0.41 **	-0.06	***
Adenylate kinase 4	NM_017135	5q33	1.17 ***	1.12 **	1.00 *	**
Allograft inflammatory factor 1	NM_017196	20p12	-0.38	-0.91	-0.16	**
Aminolevulinate synthase 2, delta	NM_013197	X	0.79 *	0.71 *	0.64	**
arrestin-E	BU671102	?	0.58 **	0.47 **	0.52	**
ATP citrate lyase	NM_016987	10q32.1	-0.28	-0.22	1.17 *	***
ATPase, Class II, type 9A	U78977	?	0.35	0.72 *	0.48	**
Cadherin 17	X78997	5q13	-0.15	-0.46 **	-1.21 **	***
Calcium binding protein P22	NM_024139	3q35	0.89 ***	0.81 **	0.82	**
carboxylesterase isoenzyme	NM_144743	19p14	0.74 **	0.70 **	0.74 *	**
Chloride ion pump-associated 55 kDa	AF332142	4q34	-0.34 **	-0.32 ***	-0.17	**
C-reactive protein	NM_017096	13q24	0.80 ***	0.58 **	0.66 ***	***
Eukaryotic translation elongation factor 2	NM_017245	7q11	0.40 **	0.30 *	0.46 **	**
Glucose-6-phosphatase (G6pc)	**NM_013098**	**10q32**	**-0.63 ** **	**-1.00 *** **	**-0.57 * **	***
Glucose-6-phosphate dehydrogenase (G6pd)	**NM_017006**	**Xq37**	**-0.43 ** **	**0.02**	**1.28**	***
Glutamylcysteine gamma synthetase light chain (Gclc)	J05181	8q31	0.50	0.63 **	0.35	
Growth response protein (CL-6)	NM_022392	4q11	-0.01	0.17	0.85 **	**
Hematopoietically expressed homeobox	**NM_024385**	**1q53**	**-0.99 *** **	**-0.40 ** **	**-0.81**	
Hemoglobin, alpha 1	NM_013096	10q12	0.32 **	0.30	-0.20	*
Hemoglobin, beta	X05080	1q22	0.51 *	0.48 *	-0.08	***
Hepatic nuclear factor 4 (alpha transcription factor 4)	NM_022180	?	1.41 **	1.07 **	0.80 *	**

Hepatocyte nuclear factor 6	NM_022671	8q24	-0.72 **	-0.50	0.56	***
Highly sim. to SMRT2 metallothionein II	**M11794**	**?**	**0.18**	**-0.77**	**-0.55**	**
Inositol polyphosphate multikinase	AY014898	20p11	-0.36	-0.42 **	-0.49 ***	***
Isocitrate dehydrogenase 1, soluble	NM_031510	9q32	0.50 **	0.42	0.52 **	*
Kinase D-interacting substance of 220 kDa	AF239045	6q16	-0.19	-0.18	0.20	***
Malic enzyme 1, soluble	NM_012600	8q31	-0.07	0.28	1.23 **	***
Metallothionein	***J00750***	**19p12**	**-0.03**	**-0.30** **	**-0.28**	
Myelin-associated/Oligodendrocytic Basic Prot-81	NM_012720	8q32	0.39	-0.63 **	-0.45 **	***
Nim3	NM_022931	5q36	-0.45 *	-0.48 *	0.86 **	***
Ornithine aminotransferase	NM_022521	1q41	0.32	-0.07	-0.31	**
Proteasome (prosome, macropain), alpha type 4	NM_017281	8q24	-0.38 *	-0.37 **	0.10	**
protein phosphatase 2, regulatory subunit A	BU671201	1q12	1.34 *	1.28 ***	1.07 *	**
Protein tyrosine phosphatase, non-receptor type 16	X84004	10q12	-0.18	0.24	0.98 *	**
Reticulocalbin 2	NM_017132	8q24	-0.53	0.58	0.82	***
Ribosomal protein L21	M27905	12p11	-0.27	-0.39 *	-0.07	**
Ribosomal protein S27a	NM_031113	14q22	-0.62 **	-0.36 **	-0.24	**
RT1 class Ib, H2-TL-like, grc region	NM_012646	20p12	-0.52 **	-0.60 **	-0.49 **	***
Septin 3	NM_019375	7q34	-0.36	-0.84 **	-0.72 **	**
Sim. to Eukaryotic translation initiation factor 3 sub.6 interact. prot	BU671701	7q34	1.04 *	0.97 **	1.19 **	**
Solute carrier family 21 (organic anions), 5	U88036	?	-0.31 **	0.05	-0.28	**
Thyroid hormone responsive protein (spot14)	NM_012703	1q32	0.22	-0.02	1.23 **	***
24-kDa subunit of mit.NADH dehydrogenase	M22756	9q37	-0.46 **	-0.32	0.09	***
6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 1	**NM_012621**	**Xq22-q31**	**-0.48 ** **	**-0.32**	**-0.68**	

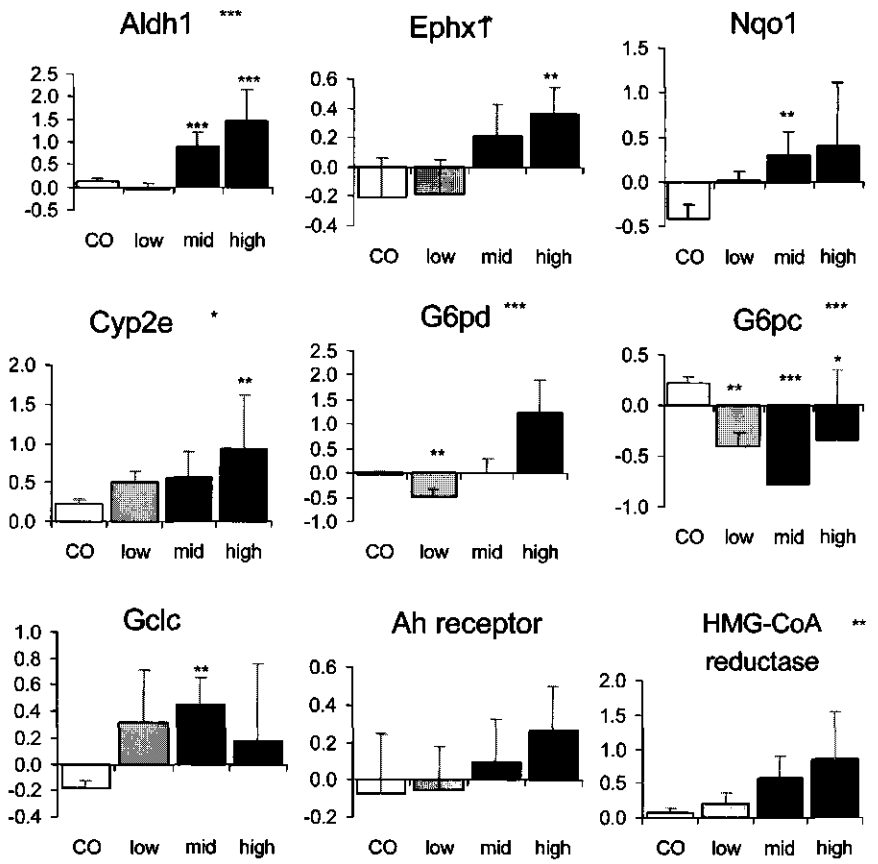

Figure V. 5. *Benzene-induced expression changes for a selection of genes.* Average expression levels (log(2)) and standard deviation (n = 4) are shown. Statistical significance is denoted as: *** $p < 0.001$; ** $p < 0.01$, * $p < 0.02$ for T-tests above the bars and for ANOVA next to the titles. *Dose levels:* CO: control, low: 10 mg/kg/day, mid: 200 mg/kg/day, high: 800 mg/kg/day. *Abbreviations:* Aldh1: aldehyde dehydrogenase 1; Ah: Aryl hydrocarbon; Cyp2e1: Cytochrome P450 enzyme 2E1; G6pc:glucose-6-phosphatase; G6pd:glucose-6-phosphate dehydrogenase; Gclc: gamma-glutamylcysteine synthetase; mEH (=Ephx1): microsomal epoxide hydrolase; Nqo1: NAD(P)H dehydrogenase, quinone 1

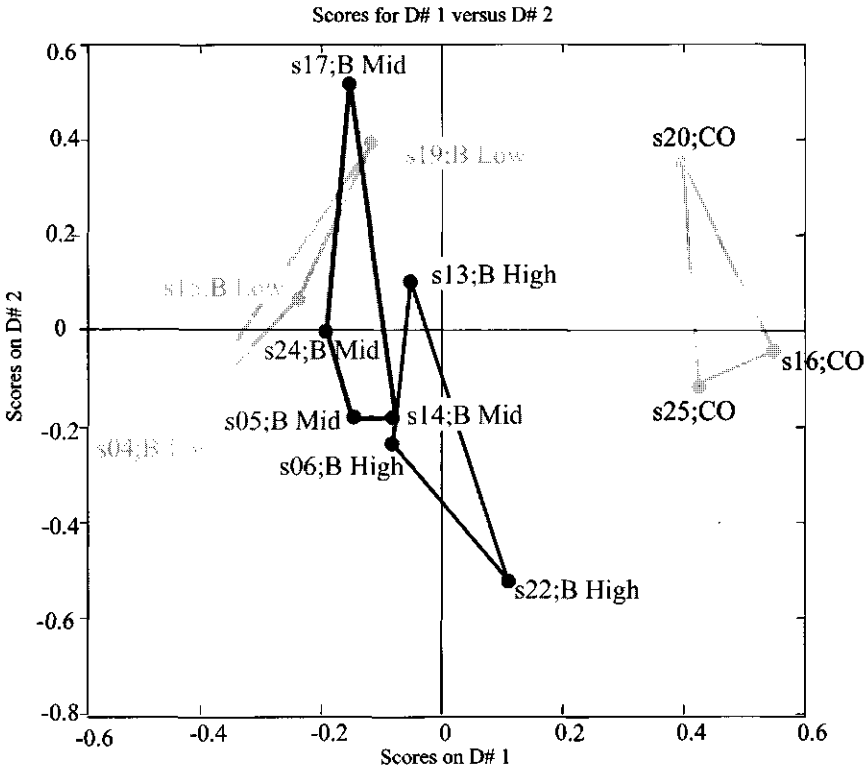

Figure V.4. *Score plot of PCDA of hepatic gene expression changes*
Score plot of the principal component analysis with discriminant (PCDA) of trancriptomics measurements of hepatic gene expression. Each symbol in the score plot represents an individual DNA microarray measurement. Per group, pooled samples of 5 rats were measured in triplicate. For each symbol, the slide identification number of the DNA microarrays is provided (s#). **CO:** controls; B: benzene; **low:** benzene 10 mg/kg/day of benzene; **mid**: 200 mg/kg/day of benzene and **high** 800 mg/kg/day of benzene.

Table V. 3a *PCDA: Genes with highest D#1 score (most reduced by benzene)*

D#1	GeneName	Acc#
1.95	Similar to SON protein (LOC304092)	AW144637
1.47	ribosomal protein L35	X51705
1.33	ATP synthase, H+ transporting, mit.F1F0 complex	D13121
1.27	Large subunit ribosomal protein L36a	NM_031105
1.23	NADH ubiquinone oxidoreductase subunit B13	NM_012985
1.21	Ribosomal protein L37	NM_031106
1.21	Ribosomal protein S24	NM_031112
1.18	Glucose-6-phosphatase	NM_013098
1.15	Ribosomal protein L35a	NM_021264
1.13	Fatty acid synthase	NM_017332

In addition to ANOVA, the multivariate statistical technique PCDA was performed to identify significantly modulated genes upon benzene exposure. Figure V.4 shows the score plot of the PCDA analysis. It demonstrates that independent of the dose, the benzene treatment altered the pattern of liver gene expression. Along the horizontal axis (D#1), the gene expression profiles from the benzene-treated rats were distinct from the controls. Thus, genes were sorted according to the scores on D#1, to reflecting their individual contribution to the differences in the gene expression profiles. Genes with the highest positive or negative contribution are listed in tables 3 a, b.

Table V.3b *PCDA: Genes with lowest score on D#1 (most induced by benzene)*

D#1	GeneName	Acc#
-2.45	apolipoprotein B (apoB)	X55969
-1.88	Retinoid X receptor alpha	NM_012805
-1.75	Apobec-1 complementation factor, APOBEC-1 stimulating protein	NM_133400
-1.49	protein phosphatase 2 (formerly 2A), regulatory subunit A (PR 65)	BU671201
-1.44	fatty acid elongase 1	NM_134382
-1.44	Hepatic nuclear factor 4 (alpha transcription factor 4)	NM_022180
-1.42	D-amino acid oxidase	BF545995
-1.4	Similar to Eif4g2 protein (LOC361628)	BU671233
-1.39	Similar to Polymerase delta interacting protein 38 (LOC287544)	BF543017
-1.37	Similar to transmembrane protein TM9SF3 (LOC309475)	AA800019

V.4 Discussion

A subacute, 28-day oral exposure study examined the toxicity of benzene in rats, and the expression of 5000 different liver genes was determined by oligonucleotide microarrays. The critical toxic effects of benzene were the decrease of white blood cells, and lower spleen and thymus weights. In addition, benzene caused a slight decrease in body weight probably caused by growth retardation through reduced food intake [150]. Benzene slightly induced the relative liver weight and hepatic GSH content, but no other hepatic effects observed with histopathology or clinical chemistry.

Urine analysis and metabolomics

Analytical methods exist to monitor benzene levels in expired breath and blood [152]; [153],[154],[155],[156]. However, the current biomarkers of exposure are not specific Because metabolites of benzene excreted in the urine could provide a characteristic profile, NMR and pattern recognition techniques were used to assess exposure to benzene in urine samples. This approach enabled to specifically and robustly distinguish urines from the different exposure levels based on metabolite profiles.

Benzene-induced gene expression changes

Many liver genes were found to be differentially expressed after exposure to benzene (table V.2A). Pattern recognition techniques enabled to distinguish treated from control samples by the hepatic gene expression profile and to identify the genes that were most significantly affected by benzene. Most likely due to the critical hematotoxic effects of benzene at the doe levels tested, a marked dose-dependency of the hepatic changes was not observed. Nevertheless, significant changes in liver gene expression were identified at all dose levels and these were categorised according to biological processes.

Biotransformation

Many biotransformation enzymes were affected by benzene. Most pronouncedly induced were several Aldhs and Adhs, Afar, and several conjugation enzymes like GSTs, UGTs and SULTs (all 2 to 2.5 fold induced). The induction of these enzymes could enhance benzene detoxification. The induction of Aldhs (figure V.5) might be a functional response to (toxic) muconaldehyde metabolites. Also enzyme inducing agents like phenobarbital induced Aldh 1 expression, via the constitutive androstane receptor (CAR) [157];[158]. Enzymes known to be crucial for benzene bioactivation, Cyp2e1 and microsomal epoxide hydrolase (Ephx1) were induced (figure V.5). This was in agreement with recently observed, 2.2-fold, induction of Cyp2e1 gene expression and protein upon 14-day benzene exposure in rats [159]. Furthermore, the absence of Cyp2e1, in knock-out mice, impaired benzene-induced toxicity [160].The present induction of Cyp2e1 might aggravate toxic effects. The importance of the Ephx1 for development of toxicity was shown in male but not in female mice [144]. Upregulation of Ephx1 could enhance bioactivation of benzene, thereby increasing the toxic effects. Hepatic Nqo1, which plays a role in detoxification of benzene metabolites was upregulated by benzene (figure V.5). Polymorphisms in Nqo1 in humans were reported to affected susceptibility to benzene poisoning [161],[162]. Nqo1 deficient mice were more susceptible to benzene-induced (hemato)toxicity [144]. Previously, the Aryl hydrocarbon (Ah) receptor was reported to be involved in benzene toxicity [163], and to induce (human) Cyp1a1, Cyp1a2 and phase II enzymes [164]. In agreement, a dose-dependent upregulation of the Ah receptor was observed, though changes were not established with statistical significance.

Oxidative stress and GSH metabolism
Because benzene metabolites are highly reactive they might induce oxidative stress Characteristic induction of heme oxygenase and ferritins, nor of other gene expression markers of oxidative stress was observed. Expression of metallothioneins was slightly reduced. Enhanced protection from reactive metabolites might come from increased hepatic GSH levels, probably a results from induction of gamma glutamylcysteine synthetase light chain (Gclc), the rate-limiting enzyme in GSH biosynthesis. Also G6pd, involved in GSH synthesis, was induced at high doses (figure V.5).

Hemoglobin metabolism
The hepatic gene expression of both hemoglobin alpha and beta subunits was induced upon exposure to 10 or 200 mg/kg/day. Concurrently, slight induction of a key enzyme in hemoglobin biosynthesis, aminolevulinate synthase 2 was observed. NADPH diaphorase (Dia1) can increase hemoglobin levels when NADPH is provided by G6pd [130]. Dia1 was slightly induced, and also G6pd was induced at high dose levels. In agreement with the gene expression changes, plasma levels of hemoglobin were elevated after exposure to benzene (figure V.2).

Glycolysis and gluconeogenesis
Enzymes important in glycolysis and gluconeogenesis were differentially expressed by benzene. G6pd was induced more than 2.5-fold by the high dose of benzene. G6pc was repressed two-fold and expression of Hnf4, which regulates G6pc and glucokinase gene expression [165]; [166] was increased by benzene. The PCDA identified Hnf4 as one of the most pronouncedly differentially expressed genes between controls and benzene-treated samples. Also the transcription factor Hnf6, with a reported role in glucokinase induction [167],[168],[169], seemed to be induced. The enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 1 involved in regulation of glycolysis was downregulated. The data suggest enhanced glycolysis and/or attenuated gluconeogenesis.

Fatty acid, cholesterol and lipid metabolism
Inductions of gene expression for many enzymes involved in cholesterol and fatty acid metabolism were measured (Table V.2B.). These were visualised using Genmapp (www.genmapp.org) in figures V.6 and V.7. Expression of a key enzyme in cholesterol metabolism, HMG-CoA reductase, was dose-dependently upregulated (figure V.5). In agreement, levels of plasma cholesterol (and phospholipids) were found to be slightly elevated in benzene-treated groups (n.s.). Both with ANOVA and PCDA, apolipoprotein B (ApoB) was identified as one of the most pronouncedly induced genes, along with Acf, the Apobec-1 complementation factor, involved in ApoB mRNA cytidine to uridine editing [170] [171]. Also noteworthy was the increased expression of the sterol regulatory element binding protein (Srebf1) at all dose levels. Srebf1 binds to regulatory element in genes involved in fatty acid metabolism, like acetyl-CoA carboxylase beta. Remarkably, expression of acetyl-CoA carboxylase beta was slightly increased at high dose exposure, but slightly decreased at mid or low dose.

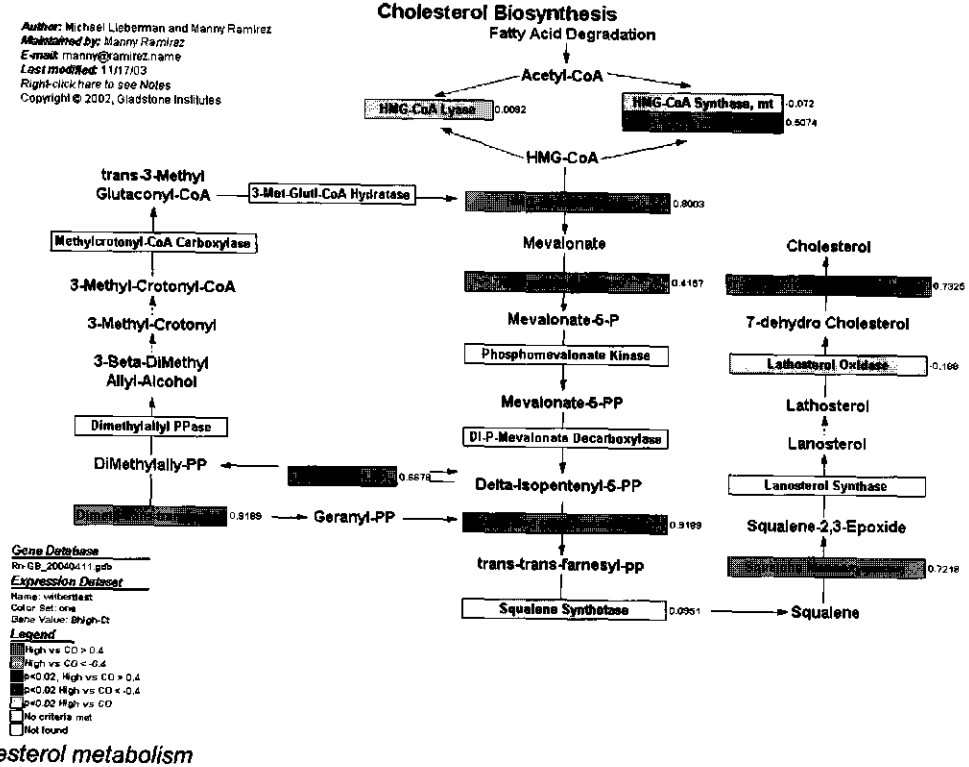

Figure V.6 *Cholesterol metabolism*

GenMapp (www.Genmapp.org) visualisation of changes in gene expression related to cholesterol metabolism. Values next to the boxes represent the average fold change in expression between the high dose exposed livers and the controls. Although none of the individual changes reached the statistical level of significance ($p < 0.02$ in ANOVA or T-test), most enzymes were slightly induced, suggesting an induction of the entire pathway of cholesterol biosynthesis. In agreement, a slight increase of plasma cholesterol levels was observed with clinical chemistry methods.

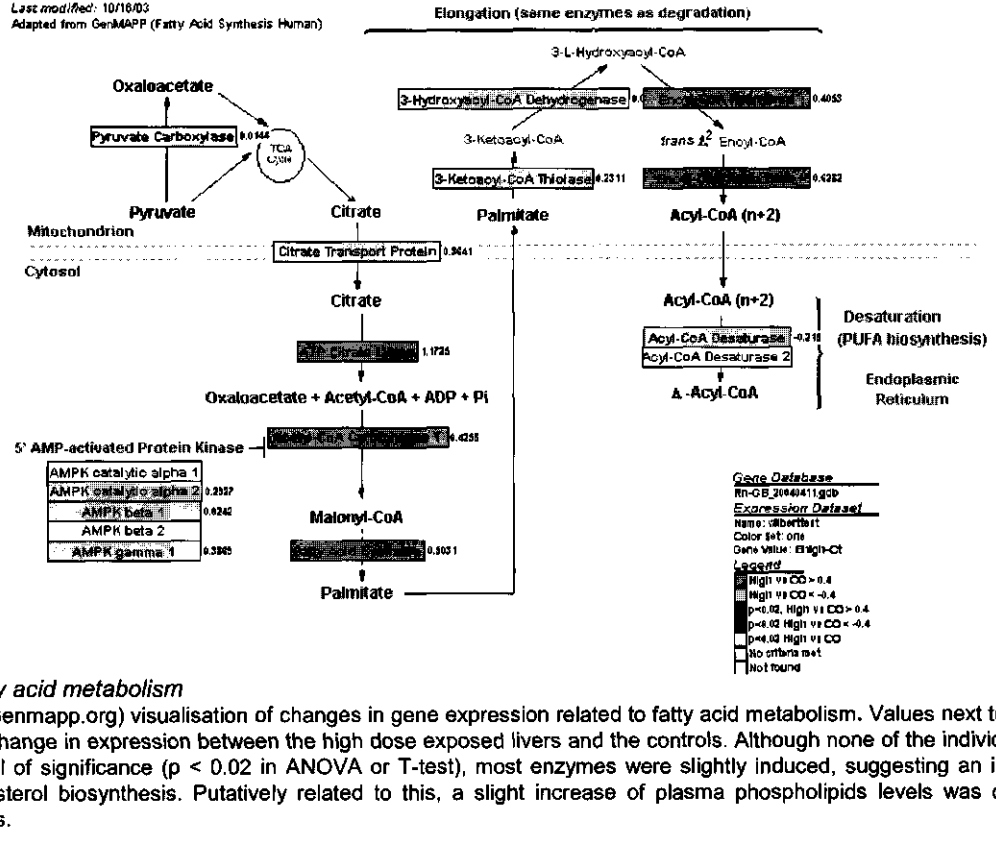

Figure V.7. *Fatty acid metabolism*

GenMapp (www.Genmapp.org) visualisation of changes in gene expression related to fatty acid metabolism. Values next to the boxes represent the average fold change in expression between the high dose exposed livers and the controls. Although none of the individual changes reached the statistical level of significance ($p < 0.02$ in ANOVA or T-test), most enzymes were slightly induced, suggesting an induction of the entire pathway of cholesterol biosynthesis. Putatively related to this, a slight increase of plasma phospholipids levels was observed with clinical chemistry methods.

Possibly associated to lipid metabolism was the slight reduction of expression of the liver-specific transcription factor Lisch7. Lisch7 binds to lipoproteins including ApoB and ApoE (rat genome database: rgd.mcw.edu). Yen and colleagues [172] speculate that Lisch7 is a rate-limiting step for the clearance of dietary triglycerides and plays a role in determining their partitioning between the liver and peripheral tissues. Lastly, an important molecular receptor, retinoid X receptor alpha, was upregulated at all dose levels. The RXR forms dimers with other nuclear receptors (eg. PPAR) and regulates expression of many genes, related to ao. fatty acid metabolism [164] including acyl CoA oxidase, which was also induced by benzene. In conclusion, the elevated plasma levels of phospholipids and cholesterol may result from enzyme induction upon benzene treatment.

Protein and RNA synthesis
Expression of various ribosome subunits and several translation factors was reduced. While, according to statistical analysis, few ribosomal subunits were significantly downregulated (table V.2), PCDA showed reduced expression of 18 ribosomal subunits amongst the 100 genes most significantly affected by benzene treatment (see table V.3). Possibly in agreement with this, less total RNA was isolated from treated compared to control liver samples.

Hematopoiesis
Benzene exposure affected the lymphocytes. Possibly in relation to this, hepatic expression of the transcription factor Hnf6, was characteristically modulated by benzene. The low and mid dose reduced its expression, while the high dose induced Hnf6. Besides its role in glycolysis, Hnf6 is involved in B lymphopoiesis in (fetal) liver [169]. Further studies will have to determine whether induction of this transcription factor is related to hematotoxicity by benzene. A gene encoding the "hematopoietically expressed homeobox" was downregulated at all dose levels. This homeobox is suggested to have a regulatory function in hematopoiesis, though its precise function has yet to be resolved.

Chromosomal aberrations
Chromosome aberrations might be reflected in expression changes of genes located on specific chromosomes. In humans, chromosomes 5 and 7 were found susceptible to aneuploidy upon benzene exposure [173]. Furthermore, the hydroquinone metabolite of benzene *in vitro*, increased trisomy and monosomy of chromosomes 7 and 8. [174]. Analysis of the chromosomal location of the genes affected by benzene (Table V.2), revealed no indications for chromosome-specificity of changes.

Table V.4 *Specificity of gene expression changes in rat*

Comparison of hepatic gene expression changes induced by bromobenzene (BB), acetaminophen (A) and benzene (B). BB and APAP were given as a single dose, and gene expression changes were followed 1-2 days after exposure (Heijne *et al.*, 2004) and (Heinloth *et al.*, 2004). Benzene (present study) was given repeatedly for 28 days.

Symbol	Gene	Acc.#[1]	Acc.#[2]	BB	A	B
Ephx1	microsomal epoxide hydrolase	NM_012844	AA900551	+	+	+
Afar	Aflatoxin B1 aldehyde reductase	NM_013215	AA923966	+	-	+
Gsta	Glutathione-S-transferase Alpha	NM_031509	AA818339	+	0/+	+
Nqo1	NADPH dehydrogenase,quinone1	NM_017000	AA899180	+	+	+
Cyp2c12	Cytochrome P450 15-beta	NM_031572	AA818124	-	-	-
Cyp2e1	Cytochrome P450, 2e1	NM_031543	AA818896	-	-	+
Aldh	Aldehyde dehydrogenases	div	div	+	0	+
Ugt	UDP-glucuronosyltransferases	div	div	+	0/+	+
G6pc	Glucose-6-phosphatase	NM_013098	AA964628	-	-	-
G6pd	Glucose-6-phosphate dehydrogenase	NM_012942	AA923963	+	0	+
Gclcl	Glutamylcysteine gamma synthetase light chain	J05181	AA965220	+	?	+
Mt	Metallothioneins	div	div	+	+	0
Timp1	Tissue inhibitor of metalloproteinase 1	ND	AA957593	+	+	?
Prdx1	peroxiredoxin1	D30035	AA875245	+	+	0
HO-1	heme oxygenase 1	NM_012580	AA874884	+	+	0
Ftl,Fth	Ferritin light and heavy chain			+	0/+	0
Orm	orosomucoid	J00696	AI029162	+	+?	ns
Mug	alpha(1)-inhibitor 3	NM_017351	AA817965	-	-	ns
	various ribosomal subunits	div	div	+	+	-
	various proteasome subunits	div	div	+	0	0/-
Hbbeta	hemoglobin beta	X05080	AA818084	+	ND	+ .. -
Hbalpha	hemoglobin alpha	NM_013096	AA819784	+	-	+ .. -
	various fatty acid metabolism genes			-	-	+
Bhmt	Betaine-homocysteine methyltransferase	NM_030850	AA901407	-	+	+
	Beta actin	NM_031144	AA859846	+	?	+

[1] Accession number of the DNA fragment used on the oligonucleotide microarrays in the B study,
[2] Accession number of the DNA fragment used on the cDNA microarrays in the BB and APAP studies

Gene expression changes in bone marrow

Recently, two reports described gene expression in bone marrow of mice exposed to benzene [175] and [176]. Faiola and co-workers tentatively concluded that upon 2-weeks of inhalatory benzene exposure, hematopoietic stem cells were not actively proliferating and the DNA repair genes were not significantly altered. Yoon and co-workers analysed exposure to benzene in wildtype and p53-knockout mice [175]. The authors suggested that benzene induced apoptosis through p53-mediated caspase 11 activation. A slight induction of Cyp2e1 and Mpo was observed in bone marrow, though the statistical significance of the changes was not indicated. Further studies are needed to assess the relation between gene expression changes in liver, blood and bone marrow, in relation to the toxic effects of benzene.

Specificity of gene expression changes: benzene compared with hepatotoxicants

Benzene induced changes in hepatic gene expression after 28-day exposure, though it remains unclear whether these are reversible or irreversible effects, leading to toxicity. To determine the specificity of the effects, they were compared to acute hepatic gene expression changes in recent studies with the known hepatotoxicants BB[2] and [3] and APAP [69]. Both BB and APAP provoked hepatic centrilobular necrosis within two days, and both dose-dependently elicited hepatic gene expression changes in common biological pathways. Also, a dose-dependent increase in relative liver weight was observed [3]. Despite of technical variation between the studies, concordance was found between many gene expression changes by BB and APAP. BB as well as APAP deregulated general processes like drug metabolism, the acute phase response, fatty acid and cholesterol metabolism, protein synthesis and glycolysis. Likewise, 28-days exposure to benzene elicited changes in most of these processes, though the effects were less pronounced, both in the number and the magnitude of the changes, and often not dependent on the dose level. Therefore, a direct comparison of hepatic gene expression changes was hampered, and the gene-by-gene comparison was limited to the most pronounced effects at the high dose levels, between effects of BB, APAP and benzene (Table V.4). Benzene and BB commonly induced the biotransformation enzymes Ephx1, Gsta, Afar and aldehyde dehydrogenases. For APAP, this response was not as pronounced. The pattern of gene induction suggests the involvement of the electrophile response element (EpRE or ARE) in the transcriptional regulation. This element was found to mediate expression of genes like Afar, Gsta, Gclc, and ferritin, peroxiredoxin1 and Nqo1 [86,177]; [124]; [87]. Oxidative stress-induced expression of HO-1, Timp1 and metallothioneins was found rapidly after single dosage of BB and APAP, but not after 28-days exposure to benzene, though this might also depend on the duration of the treatment. Six hours after BB dosage, hepatic GSH levels were largely depleted, but they were restored after 24 hours, most likely through the induction of the Gclc enzyme. Also benzene induced Gclc and, in agreement, GSH levels were elevated after 28 days of exposure. G6pd was induced and G6pc repressed in common by BB, APAP and benzene. The Cyp2c12 gene was commonly decreased by all compounds, and Cyp2e1, decreased by BB and APAP, was induced by benzene. In summary, benzene, which induced liver enlargement but no histopathological effects, elicited several gene expression changes in common with known hepatotoxicants. However, there was a clear difference in the number, the magnitude of the hepatic gene expression changes and the dose-dependency.

Conclusion

In summary, without provoking overt hepatotoxicity, benzene changed expression of many liver genes in rats. In addition to gene expression profiling, urine metabolite profiling (metabolomics) proved useful to specifically and robustly assess benzene exposure. Benzene altered expression of genes with putative implications for (its own) biotransformation, fatty acid, cholesterol and hemoglobin metabolism, and other processes. Hepatic gene expression changes were related to observed increases in relative liver weight and GSH levels, and possibly also to toxic properties of benzene. The specificity of liver gene expression changes was determined in a comparison of the benzene-induced effects with those of hepatotoxicants BB and APAP. Several genes were transcriptionally regulated in common by the chemicals, such as through suggested involvement of the electrophile response element. However, there was a clear difference between benzene and the model hepatotoxicants in the number, the magnitude of the hepatic gene expression changes and the dose-dependency of the effects.

Acknowledgements

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Chapter VI

Toxicogenomics analysis of liver gene expression in relation to subacute toxicity in rats exposed to trichloroethylene

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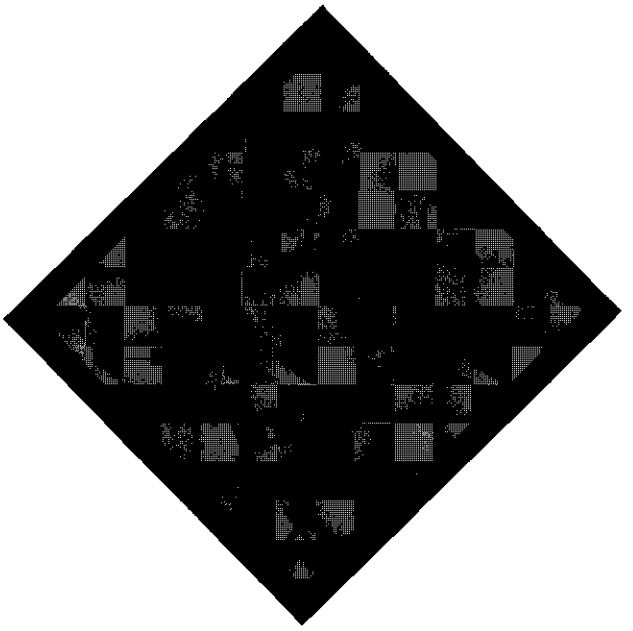

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Abstract

This study analysed hepatic gene expression changes and subacute toxicity markers in rats after 28-day exposure to trichloroethylene (TCE). TCE is a widely used chemical, nephrotoxic and putatively hepatotoxic. Hepatic metabolism determines the toxicity of TCE. The aim was to characterise the hepatic gene expression changes in relation to hepato-pathological changes induced by TCE. Gene expression changes were determined with DNA microarrays in livers of male F344 rats treated for 28 days with TCE, and subacute toxicity was assessed after exposure to 50, 500 and 2000 mg/kg/day. Metabolite profiles of urine were analysed in a metabolomics approach, to specifically and robustly assess exposure to TCE, even at 50 mg/kg/day.

Conventional toxicology revealed nephrotoxicity, but no hepatotoxicity besides increased relative liver weight from 500 mg/kg/day. Transcriptomics revealed dose-dependent hepatic gene expression changes, from 50 mg/kdg/day. TCE markedly induced expression of biotransformation (ao. Cyp2e1, Gsta, Aldh1) and beta oxidation enzymes, and many others. The changes were related to bioactivation and detoxification of TCE, with possible consequences for nephrotoxicity and carcinogenicity. For example, elevated hepatic glutathione biosynthesis and induction of glutathione-S-transferases could enhance formation of nephrotoxic metabolites. Decreased hepatic expression of alpha 2u-globulin explained why TCE did not cause hyalin nephropathy. TCE modulated several genes in common with hepatotoxicants that caused liver necrosis or peroxisome proliferation, and with benzene, though many transcriptome changes were characteristic for TCE exposure. In conclusion, this study characterised TCE-induced hepatic gene expression changes at doses that induced weight increase but no other hepatotoxic effects.

VI.1 Introduction

Transcriptomics methods, that measure gene expression with DNA microarrays, enabled to study cellular mechanisms in great detail. A variety of applications of these methods in toxicology (toxicogenomics) has been reported [34],[21],[22],[27]. In our laboratory, gene expression changes in liver were characterised in detail after exposure to the hepatotoxicant bromobenzene (BB) [2];[3]. Simultaneously, Heinloth and colleagues have analysed gene expression changes after a high dose of acetaminophen (APAP) [69]. BB and APAP are well-known hepatotoxicants that caused hepatic necrosis, increased the relative liver weight and, in parallel, changed the expression of many hepatic genes. These changes in gene expression were related to, a.o., biotransformation, glutathione metabolism, protein synthesis and proteolysis, fatty acid and cholesterol metabolism, oxidative stress and the acute phase response.

To further investigate the specificity of hepatic gene expression changes in relation to hepato-pathological changes, we used transcriptomics to study the effects of benzene [5]and trichloroethylene (TCE) (this paper), and compared the effects with those of the model hepatotoxicants. The liver plays a central role in the biotransformation, ultimately leading to bioactivation and detoxification of benzene as well as TCE. TCE is a solvent applied in large quantities to produce other chemicals, and consumer products such as paint removers, adhesives, degreasers and typewriter correction fluid contain TCE. Human exposure may occur through contaminated water or ambient air. The metabolism and toxicity of TCE have been reviewed in detailed [178];[179];[180]. TCE is not considered a (model) hepatotoxicant. Nevertheless, in parallel to nephrotoxicity, TCE induced effects in liver such as weight increase [181];[128] and increased peroxisomal β-oxidation activity [182];[183]. TCE induced tumours in rats and mice, but there is an ongoing debate about its (nephro)carcinogenicity in humans [184-186].

The purpose of the present study was to investigate the specificity of changes in hepatic gene expression, upon exposure to the industrial chemical TCE. For this purpose, rats were exposed to trichloroethylene for 28 days, and subacute toxicity was determined with conventional toxicological methods, while transcriptome changes in the liver were determined with oligonucleotide DNA microarrays. In parallel with conventional urine analysis, a metabolomics approach with NMR and pattern recognition was used to analyse the urine, to specifically and robustly demonstrate exposure to TCE in urine samples. The effects of TCE at the hepatic transcriptome level were compared with gene expression changes elicited by model hepatotoxicants that cause hepatic necrosis, and with effects of benzene [5]. Because earlier studies showed peroxisome proliferation upon exposure to TCE [182], the data were also compared to effects of compounds inducing peroxisome proliferation.

VI.2 Materials and Methods

TCE was obtained from Aldrich Chemical Co. (Milwaukee, USA), and corn oil from Remia (Den Dolder, The Netherlands). Male F344 rats (CDFF® (F344)/CrlBR) from Charles River Wiga GmbH (Sulzfeld, Germany). Were kept in accordance with the general principles governing the use of animals in toxicity experiments of the European Communities (Directive 86/609/EEC) and Dutch legislation (The Experiments on Animals Act, 1997). The rats were housed in macrolon cages with stainless steel grid covers and wood shavings as bedding material, five rats per cage, in a room maintained at 22 ± 2°C with a relative humidity of 30-70% and a 12 h light/dark cycle. Throughout the study, feed (a powdered, cereal-based, closed-formula rodent diet (Rat and mouse No. 3 Breeding diet; RM3) from Special Diets Services (Witham, England)) and tap water were freely available. Following an acclimatization period of 8 days, the 5-week old rats were allocated randomly to groups of five, in such a way that the mean body weights were about the same in all groups.

Administration route, duration and dose levels
TCE was dissolved in corn oil and administered by oral gavage in a volume of 5 ml per kg body weight at dose levels of 50, 500 and 2000 mg/kg/day, once daily for 28 consecutive days, for the last time on the day before scheduled sacrifice. The highest dose level was intended to induce toxic effects but no severe suffering, death or marked growth retardation. The low- and mid-dose levels were selected to demonstrate dose-effect relationships, and no toxic effects at the lowest dose level [178] Controls were dosed with corn oil only. The dose volumes were adjusted twice per week for changes in body weight. Fresh TCE solutions were prepared once per week and stored at 2-10°C in portions sufficient for one day.

Observations and analyses
General clinical observations were made daily before and after dosing. The rats were weighed twice per week and at final necropsy. Food consumption was measured per cage, over successive periods of 7 days (wk 1-3) or 4 days (wk 4). Water consumption was measured per cage, on three consecutive days in wk 1-3. Water consumption of individual animals was measured during the 24 h urine collection period in wk 4. Routine neurobehavioral functioning was evaluated on day 22 before the daily dosing, according to Heijne *et al.*[5].

Urine analysis and metabolomics
Urine was collected at the end of the fourth week of treatment. The rats were kept separately in metabolism cages for two consecutive days. After one day of acclimatisation, 24 h urine samples were collected, in ice-cooled containers. Each sample was characterised with respect to appearance, volume, density (Bellingham and Stanley refractometer), total protein (Cobas-Bio centrifugal analyser), glucose, creatinine, alkaline phosphatase (ALP), gamma glutamyl transferase (GGT), lactate dehydrogenase (LDH) and N-acetyl-ß-glucosaminidase (NAG) (Automatic analyser model 911 from Hitachi, Japan). Protein, glucose, creatinine and the enzyme activities were expressed as units excreted per 24 h by multiplying the concentrations measured by the volume of the urine produced.

The urine samples were also analysed with a metabolomics approach, by NMR spectroscopy according to Lamers *et al.* [126] and Heijne *et al.* [4]. The multivariate statistical technique PCDA was used to determine differences in the NMR profiles of the metabolites in urine. PCDA [147] is a semi-supervised method based on principal component analysis that uses the dose levels as a discriminant to optimise the contrast between the treatment groups. After calculations, the differences between the individual profiles were visualised in a score plot. Along the axis of the score plot, the D#1 and D#2 values were plotted. The score plot displayed every individual profile (in this case NMR spectrum) as one dot, and the distance between the dots reflects the degree of similarity between the individual profiles.

Haematology
At final necropsy, blood samples were collected from the abdominal aorta using K_2-EDTA as anticoagulant. Each sample was analysed for haemoglobin, packed cell volume, red blood cell count,

total white blood cell count, thrombocyte count (Sysmex K-1000 Haematology Analyser from Toa Medical Electronics Co., Ltd, Japan), differential white blood cell counts (microscopic examination of stained blood smears), and prothrombin time (Normotest, Nyegaard and Co. A/S, Norway). Mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration were calculated.

Clinical chemistry
At final necropsy, blood samples were collected from the abdominal aorta in heparinised tubes. The samples were centrifuged and the plasma was analysed for ASAT, ALAT, ALP, GGT, LDH, total protein, albumin, glucose (non-fasting), urea, creatinine, total bilirubin, total cholesterol, triglycerides and phospholipids (Automatic analyser model 911 from Hitachi, Japan). The albumin/globulin ratio was calculated.

Hepatic GSH and Cyp
Frozen liver tissue was homogenised in 0.01 M Tris-HCl/0.14 M KCl, pH 7.4 buffer (1:3 w/w) using a Potter-Elvejhem tissue homogeniser. Post-mitochondrial supernatant (S9-mix) was prepared by centrifugation at 10 000 g for 20 min. An aliquot of the S9-mix was stored at <-20°C until analysed for GSH. Microsomes for Cyp analysis were prepared by ultracentrifugation (100 000 g for 75 min) of S9-mix. The microsomal pellet was resuspended in homogenization buffer and centrifuged for 60 min at 100 000 g. The washed microsomes were suspended in homogenization buffer and stored at <-70°C until analysis. The S9-mix and microsomes were analysed for protein content using the Bradford method. Total glutathione (GSH) was measured according to Anderson *et al.* [81] Total hepatic Cyp protein contents determined according to Rutten *et al.*, [148].

Organ weights and pathology
At the end of the treatment period, the rats were killed by exsanguination from the abdominal aorta under CO_2/O_2 anaesthesia and examined grossly for pathological changes. The kidneys, liver, prostate, spleen, testes and thymus were weighed. After fixation in a 4% neutral buffered solution of formaldehyde, the kidneys, liver, spleen and thymus were processed, embedded in paraffin, sectioned at 5 µm, stained with haematoxylin and eosin, and examined by light microscopy. In addition, parts of these organs were snap-frozen in liquid nitrogen and stored at < -70°C.

Statistical analysis of conventional toxicity parameters
Body weights were evaluated by one-way analysis of covariance (covariate: body weight on day 0) followed by Dunnett's multiple comparison tests. The continuous neurobehavioral parameters (grip strength, landing foot splay), water consumption measured per animal, red blood cell and clotting potential variables, total white blood cell counts, absolute differential white blood cell counts, clinical chemistry values, routine urinary parameters (except for appearance), organ weights and hepatic levels of Cyps and GSH were evaluated by one-way analysis of variance followed by Dunnett's multiple comparison tests. Rank order neurobehavioral data (gait score, arousal, reflexes), relative differential white blood cell counts, and urinary appearance were evaluated by Kruskal-Wallis non-parametric analysis of variance followed by Mann-Whitney U-tests. Histopathological findings were analysed by Fisher's exact probability test. All analyses were two-sided.

Transcriptome analysis: liver RNA extraction
Liver samples were pulverised with mortar and pestle in liquid nitrogen before isolation of total RNA. Total RNA was extracted from liver homogenate using Trizol according to the manufacturer's protocol (Invitrogen, Breda, The Netherlands) and further purified using the RNEasy RNA purification kit, including a DNA digestion by RNAse-free DNAseI (Qiagen, Westburg B.V., Leusden, Netherlands). RNA was checked for purity and stability by gel electrophoresis and the concentration was calculated from the OD260 as determined spectrophotometrically.

Transcriptome analysis: design
A referenced design was used for microarray hybridisation, to account for dye-dependent effects. Tester samples contained equivalent amounts of total RNA of the treated rat livers, pooled per dose group. These were fluorescently labelled with Cy5, while reference material, obtained from pooled RNA of the livers of the control rats, was labelled with Cy3.

Transcriptome analysis: oligonucleotide microarrays
Oligonucleotide microarrays were produced in collaboration with the University Medical Center Utrecht Genomics Laboratory [149]. The method adapted form DeRisi [48], was described before, [2]. Briefly, about 5000 different oligonucleotide fragments (Qiagen Operon, Westburg B.V., Leusden, The Netherlands) were arrayed in duplicate on glass slides. Control cDNAs were included with no significant homology to (known) rat cDNAs as well as spots with only buffer. *In vitro* transcription reactions with indirect fluorescence labelling were performed according to [2]. After hybridisation (16 hours at 42°C), slides were washed and scanned with a Packard Scanarray confocal laserscanner, using different lasers for both the cy3 (wavelength 550nm) and cy5 (wavelength 650nm) channel. Two TIFF images per slide were saved for analysis using Imagene 5.0 (Biodiscovery Inc., El Segundo,CA, USA) Settings were applied to automatically flag weak or negative signals and spots with a non-homogenous signal. Excel (Microsoft Corporation, Redmond,USA) and SAS (SAS, Cary, USA) were used to further process and analyse the data.

Transcriptome analysis: DNA microarray quality criteria
Technical replicate hybridisations of the samples were performed to obtain, 4 microarrays per sample according to our stringent quality criteria. These criteria 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 hybdridised microarray with respect to others of the experiment.

Transcriptome analysis: DNA microarray data processing and statistical analysis
After image analysis, the local background intensity value was subtracted from the signal intensity for each spot. Background intensities outside the cDNA spots were very low and homogeneous. Fluorescence intensity in control spots was used to determine a minimal signal intensity threshold value of 1.5 for the two channels. Flagged spots and controls were excluded from further interpretation, as well as genes for which less than 70 % of the microarrays delivered a reliable signal. After this stringent selection, around 40% of the genes on the microarray were kept. Ratios of the background-corrected intensities of tester over reference were calculated and, to account for technical variations introduced during labelling or hybridization, data were normalised using the lowess normalization algorithm [103] in the SAS program. Data were log(2) transformed, and excluded values were replaced by 0. Statistical analysis of the differences between the treatment groups was performed with ANOVA in SAS. Changes with an ANOVA p-value < 0.01 were considered statistically significant.
In parallel with ANOVA, multivariate statistical techniques for pattern recognition were applied to the log(2) ratios. Unsupervised PCA was used, as described before [2], to discern transcript profiles from rats exposed to the different concentrations of TCE and controls. PCA indicated that, independent of the treatment, two microarrays were aberrant from the others, even though the technical quality criteria were met. These slides, s91 and s92 were rejected from PCDA. The PCDA was performed as described above for the urine metabolomics data, and a score plot was drawn. The genes with the largest significance for the differences between samples treated with TCE and controls were found by ranking of the genes according to D#1.

VI.3 Results

This study analysed subacute toxicity in rats exposed to TCE and related the results of hepatic transcriptomics analysis to the outcome of conventional toxicity tests.

Subacute toxicity of TCE

No TCE-related mortality occurred. The daily general clinical observations before dosing and the neurobehavioral assessment showed no treatment-related changes. After dosing, animals given TCE at 50 mg/kg/day showed no abnormalities. Rats given 500 or 2000 mg/kg/day showed on many days the following, transient symptoms: walking with hunched back, excited, lethargy and ataxia. Blepharospasm, salivation or smacking movements were observed incidentally. Rats treated with 500 and 2000 mg/kg/day consumed slightly more water (n.s.) and food consumption was decreased in the rats given 2000 mg/kg/day. These rats had reduced growth rate and terminal body weights (table VI.1).

Table VI.1. *Terminal body weights (g) and relative liver and kidney weights.*

Mean values (mg/kg BW) ± s.e.m. are shown per group, n = 5.

Treatment	Dose	Body weight	Rel. liver	Rel. kidney
Control	**0**	210.7 ± 7.2	40.1 ± 0.6	7.15 ± 0.07
TCE	**50**	198.7 ± 4.3	40.8 ± 0.8	7.19 ± 0.21
TCE	**500**	206.3 ± 3.0	45.6 ± 1.6**	7.63 ± 0.14
TCE	**2000**	177.3 ± 4.2 **	49.9 ± 0.5**	7.55 ± 0.16

Statistical significance: One-way ANOVA + Dunnetts tests: *: $p < 0.05$; **: $p < 0.01$

Organ weights and pathology

Animals given TCE had increased relative liver weights at 500 and 2000 mg/kg/day (Table VI.1). At 2000 mg/kg/day, statistically significantly lower absolute kidney, spleen and thymus weights and increased relative testes weights were found, though these effects were considered secondary to the lower body weights in this group. Histopathological examination showed karyomegaly in the kidneys of all rats treated with 2000 mg/kg/day of TCE. Increased hyaline droplet nephropathy was observed in the controls (incidence 3/5) and low dose group (4/5) but not in the mid and high dose group. The other organs showed no TCE-related histopathological changes.

Haematology

Haematology revealed no significant changes related to treatment with TCE. Differential white blood cell counts showed a slight shift from lymphocytes to neutrophils after 500 and 2000 mg/kg/day (n.s.).

Clinical chemistry and hepatic GSH and Cyp measurements

TCE significantly decreased plasma glucose and increased phospholipids at 2000 mg/kg/day (Figure VI.1A and B). Albumin was increased at 500 mg/kg/day, and the albumin/globulin ratio was dose-dependently increased at 500 and 2000 mg/kg/day (Figure VI.1C). Total hepatic glutathione (GSH) was dose-dependently increased at 500 and 2000 mg/kg/day (Figure VI.1D). Hepatic Cyp contents showed no significant differences between animals given TCE and controls.

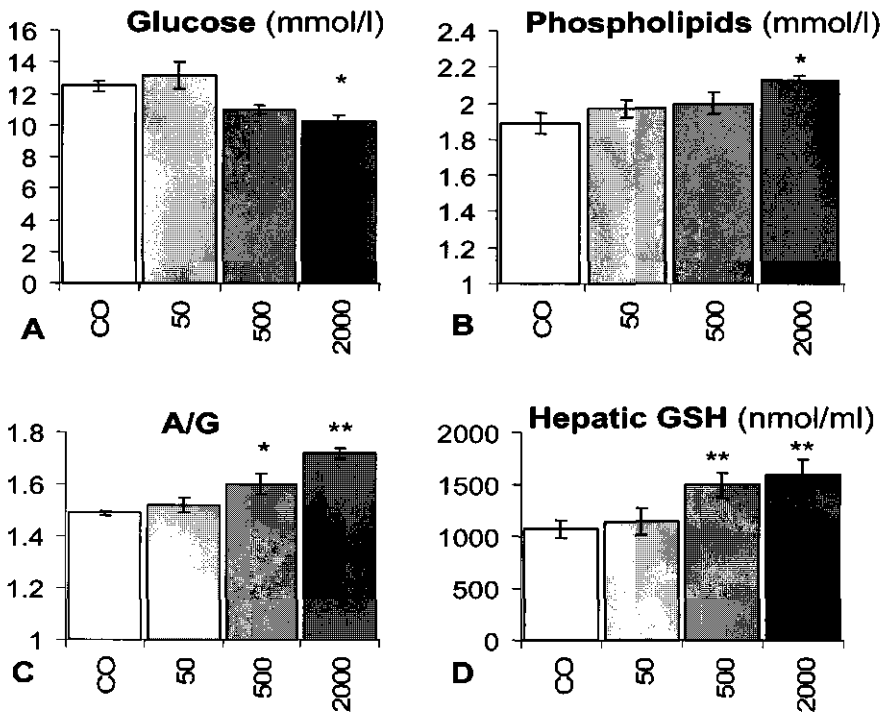


Figure VI.1. *Plasma and liver parameters determined in controls and in rats exposed to TCE at dose levels of 50, 500 and 2000 mg/kg/day.*
Panel A. Mean glucose levels ± s.e.m. in plasma (mmol/l), **Panel C.** Mean phospholipids levels ± s.e.m. in plasma (mmol/l), **Panel C.** Mean albumin to globulin (A/G) ratio ± s.e.m. in plasma, **Panel D.** Mean hepatic total GSH contents ± SD (nmol/ml). Statistical significance is denoted as follows: *: $p < 0.05$; **: $p < 0.01$.

Urine analysis and metabolomics

Rats given TCE at 2000 mg/kg/day produced more urine, with a decreased density and decreased concentrations of creatinine, glucose, protein and enzymes (ALP, GGT, LDH)). When expressed per 24 h or related to the excretion of creatinine, the excretion of protein and GGT (n.s.) was lower, whereas the excretion of NAG was higher than in controls, after treatment with 2000 mg/kg/day of TCE.

Urine samples were also analysed with NMR spectroscopy and PCDA, and results were shown in a score plot (figure VI.2). The plot indicated that metabolite profiles of urine from rats exposed to TCE differed from controls, in a dose-dependent manner. The profiles of samples from the same treatment group were more similar to each other than profiles between groups. The TCE treatment effect exceeded the interindividual and technical variation, even after exposure to low dose levels (50 mg/kg/day). The aim here was to obtain discriminative profiles, rather than to identify individual metabolites in urine. Nevertheless, the NMR spectra revealed at a glance that the differences were predominantly caused by

signals with a chemical shift of around 3.5 ppm, presumably corresponding to phase II conjugates of TCE biotransformation, such as N-acetylcysteine and glutathione conjugates of TCE. Also TCE and ethanol peaks were markedly elevated by the treatment. These NMR signals were part of a characteristic pattern of many signals that dose-dependently changed after TCE treatment, compared to controls.

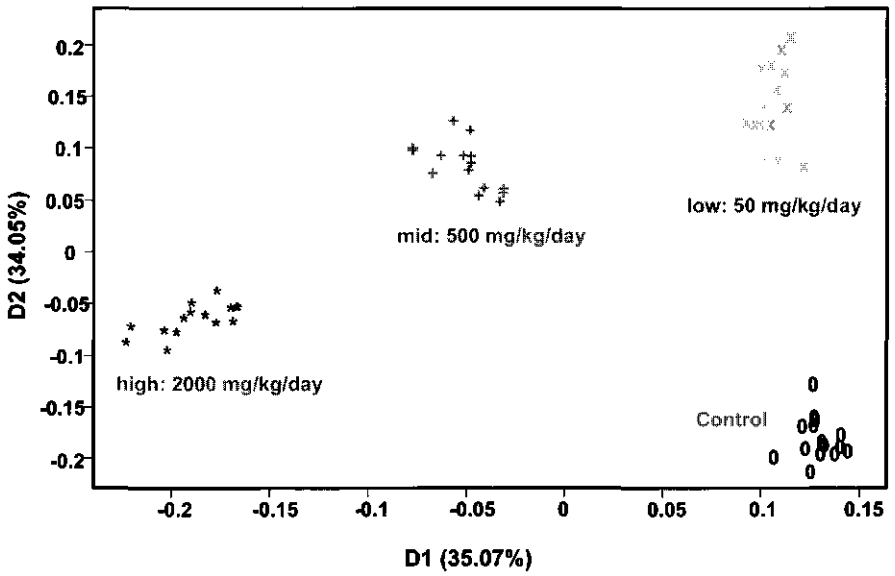


Figure VI.2. *Metabolomics – PCDA Score plot*
Score plot of principal component analysis with discriminant (PCDA) of NMR spectra, reflecting the metabolite profiles of urine. Each symbol in the score plot represents an individual NMR spectrum. Per group, samples of 5 rats were measured in triplicate. The symbols represent: 0: controls; x: exposure to low dose of TCE (50mg/kg/day); + : mid dose of TCE (500mg/kg/day); and *: high dose of TCE (2000 mg/kg/day).

Hepatic transcriptome analysis

Liver genes identified by DNA microarrays to change upon treatment with TCE were categorised and listed in the tables VI.2 A-E. For these tables, mean log(base2) transformed gene expression levels were calculated, corrected for the expression levels in the controls. Genes that altered expression upon treatment with TCE were categorised according to biological processes like drug metabolism, GSH metabolism and oxidative stress, fatty acid and cholesterol metabolism and protein synthesis & proteolysis. For a selection of genes, histograms show the mean gene expression levels and the statistical significance of the changes (figure VI.4).

A dose-related increase was found in the number of genes that were differentially expressed upon exposure to TCE (figure VI.3). The high dose altered levels of 126 genes, the mid dose 94 and the low dose 57 genes. Also the magnitude of the changes depended on the dose level. After exposure to TCE at the high dose, up to five-fold changes in mRNA levels were measured compared to control levels, the mid dose elicited changes up to three-fold, and at the low dose, changes were two-fold or less.

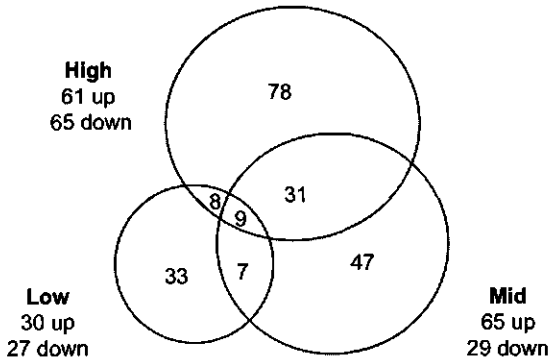
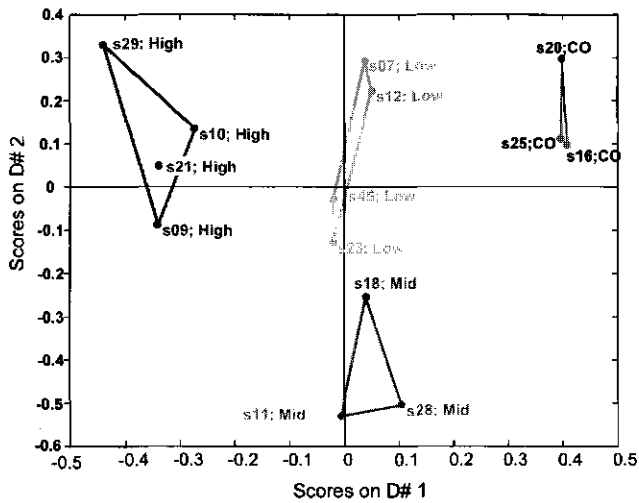


Figure VI.3. *TCE-induced gene expression changes*
Diagram of hepatic gene expression changes, compared to controls, after 28-day exposure to TCE at dose levels of 50, 500 and 2000 mg/kg/day. Only genes were counted with changes with a $p < 0.01$ in the statistical tests. If only changes were selected with a $p < 0.001$, treatment with 50, 500 and 2000 mg/kg/day altered expression of 8, 21 and 34 genes, respectively.

In parallel to ANOVA, multivariate statistical methods were applied to assess the effects of TCE on the individual hepatic gene expression profiles. Figure VI.5 shows a score plot of the PCDA of gene expression profiles. Even at 50 mg/kg/day, the treatment elicited effects larger than the technical variation. Along the horizontal axis, (D#1), the low and mid dose groups were distinct from controls and the high dose increased this distinction. The vertical axis, (D#2), was found to primarily explain the technical variation. The relative contribution of the individual genes to the TCE-induced differences in the profiles were shown by ranking the genes according to D#1. Most of the genes with a high contribution were also identified to change highly significantly in the ANOVA. To illustrate this overlap, genes that appeared in the top or bottom 100 of the ranking according to D#1 in the PCDA, were shown in boldface in the tables with ANOVA results (tables 2A-E).



Previous page **Figure VI.5.** *Analysis of hepatic gene expression profiles*
Score plot of principal component analysis with discriminant (PCDA) of measurements of hepatic gene expression. Each symbol represents a DNA microarray measurement. Per group, pooled samples of 5 rats were measured in triplicate. The identification number of the DNA microarrays is provided (s#). **CO:** controls; **low:** TCE 50 mg/kg/day of TCE; **mid**: 500 mg/kg/day of TCE and **high** 2000 mg/kg/day of TCE

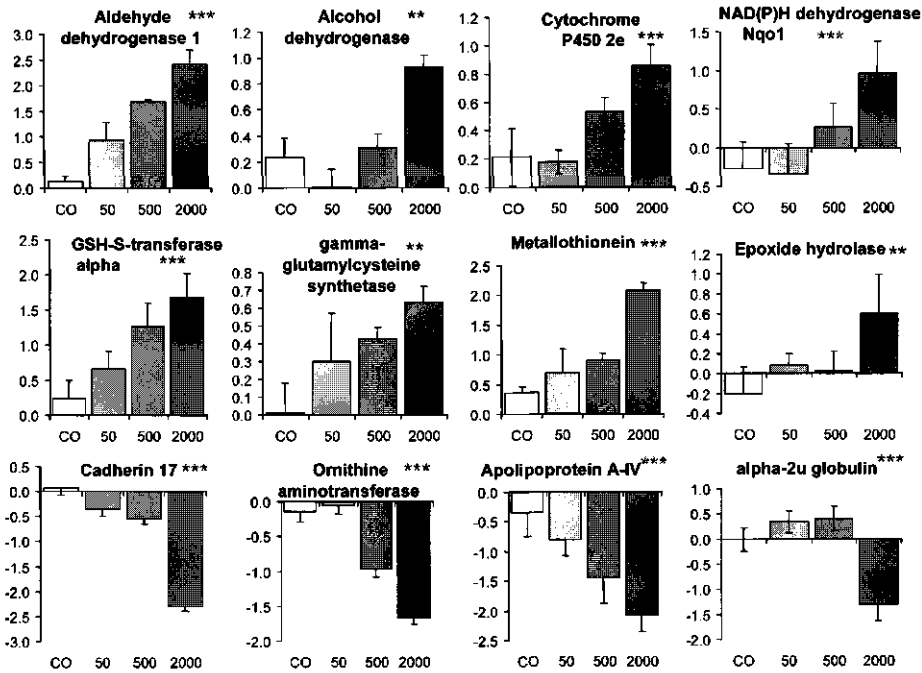


Figure VI.4. *Selection of gene expression changes induced by TCE.*
Group mean log(2) transformed gene expression levels as, measured by DNA microarrays, are shown for controls (CO) and samples exposed to TCE at dose levels of 50, 500 and 2000 mg/kg/day. Error bars represent standard deviations. Statistical significance as determined by ANOVA is denoted as follows: **: $p < 0.01$, ***: $p < 0.001$.

VI.4 Discussion

The effects of TCE on hepatic gene expression in rats exposed to TCE for 28 day were investigated with DNA microarrays. The results were related to (conventional) assessment of subacute toxicity. To delineate the specificity of hepatic transcriptome effects induced by TCE, a comparison was made with reported gene expression changes induced by hepatotoxicants that cause hepatic necrosis or peroxisome proliferation.

Subacute toxicity of TCE

The conventional toxicity endpoints demonstrated no TCE-related changes at 50 mg/kg/day. The effects observed at higher dose levels were comparable to those in earlier studies [187],[178]. TCE exposure resulted in lower terminal body weights at 2000 mg/kg/day. At this dose level, nephrotoxicity (karyomegaly), and increased excretion of NAG in urine were found. Plasma glucose was decreased and phospholipids and the A/G ratio increased, possibly associated to changes in the liver. Besides relative liver weight and GSH increases at 500 and 2000 mg/kg/day, no indications of TCE-induced hepatotoxicity were observed. Conventional toxicity markers did not reveal TCE-related effects at 50 mg/kg/day.

Urine analysis and metabolomics

The various routes of biotransformation and elimination of TCE result in characteristic metabolite contents in urine, which have been thoroughly studied [185];[180]. The current analytical methods to monitor TCE levels in human urine and blood have limited value, with low specificity and high variation between individuals and sexes [178]. These limitations might be overcome by measurement of profiles of hundreds of metabolites, that are likely more specific and robust markers than single metabolite levels. Without the aim to identify individual metabolites and resolve routes of biotransformation, we measured profiles of metabolites in urine as potential markers of TCE-exposure. While, conventional urine analysis showed some effects at high doses exposure (2000 mg/kg/day), metabolomics analysis demonstrated that the metabolite profiles of urine changed dose-dependently, from the lowest dose tested in this study (50 mg/kg/day). A characteristic pattern of multiple NMR signals was found, that dose-dependently changed. A few signals were identified, corresponding to TCE, ethanol and various N-acetylcysteine and glutathione conjugates of TCE. In conclusion, NMR profiling provided a sensitive, specific and robust method potentially useful to determine exposure to TCE in urine.

Hepatic transcriptomics analysis

This study demonstrated that TCE dose-dependently changed the expression of genes, both in the number and magnitude of changes. While conventional toxicity markers demonstrated effects in liver from 500 mg/kg/day (increase of relative liver weight and GSH), transcriptomics enabled to sensitively detect significant effects at the lowest dose level tested (50 mg/kg/day). Analysis of gene expression changes was performed with two different statistical methods, ANOVA and PCDA. While a large overlap was found, there were also genes with a significant change in only one of the methods. Nevertheless, both methods identified the same biological processes to be involved in the response to exposure to TCE. For instance, both methods identified ribosomal and proteasomal subunits to be induced, although some different subunits were significant in either ANOVA or PCDA. Thus, univariate

(ANOVA) and multivariate (PCDA) statistical methods were complementary to each other in the identification of relevant TCE-modulated genes.

Gene expression changes related to processes that could be relevant in the response to TCE are described in more detail below. These processes were drug metabolism, GSH metabolism and oxidative stress, peroxisomal beta oxidation, fatty acid and cholesterol metabolism and protein synthesis & proteolysis. Other biological processes that were affected include the acute phase response, apoptosis, signal transduction, cell adhesion, cell structure and cytoskeleton, as listed in table VI.2E.

Drug metabolism, oxidative stress and GSH metabolism

Many enzymes involved in drug metabolism were induced by exposure to TCE (table VI.2A). Most pronouncedly changed were aldehyde dehydrogenases (Aldh), Afar, alcohol dehydrogenases (Adh), and GSH-S-transferases (Gst) (figure VI.4). Also UDP-glucuronosyltransferases (Ugt) were induced, which convert TCE and its metabolites to more water-soluble conjugates. While in general, conjugation detoxifies hazardous metabolites in the liver, GSH-conjugates of TCE can induce toxicity in the kidneys upon bioactivation by γ-glutamyltransferase and dipeptidases [186]. The induction of Cyp2e1 (figure VI.4) has important implications for TCE biotransformation, being the most important enzyme for oxidation of TCE.

Metabolites of TCE might be highly reactive and increase reactive oxygen species formation. Indeed, genes involved in oxidative stress like several metallothioneins (figure VI.4) and peroxiredoxin1 were significantly induced. Oxidative stress is known to be accompanied by the induction of heme oxygenase and ferritins. However, after 28-days exposure to TCE, these genes were not found to be significantly upregulated. The induction of Nqo1 (figure VI.4) might enhance detoxification of reactive species, while protection is also provided by hepatic GSH. GSH levels may be elevated by induction of the rate-limiting enzyme for the biosynthesis of GSH: both the light chain (Gclc) (figure VI.4) and the regulatory chain (Gclm) of gamma glutamylcysteine synthetase were upregulated by TCE. The induced enzyme G6pd converts $NADP^+$ to NADPH, used by GSH reductase to restore reduced GSH levels. Hepatic GSH levels were found dose-dependently elevated in rats exposed to TCE for 28 days, indicating that more GSH was synthesised than consumed in conjugation reactions catalysed by Gsts. While Gstm3 gene expression was reduced at high dose exposure, Gsta1, the most abundant GST in liver was dose-dependently induced by TCE.

Table VI.2A. *Genes modulated by trichloroethylene, related to hepatic drug metabolism, GSH metabolism and oxidative stress.*
The level of statistical significance (Student's T-tests) is indicated next to the mean log(2) transformed fold changes, and listed in the last column for the ANOVA (**A**) (*: $p < 0.02$, **: $p < 0.01$, ***: $p<0.001$). Genes in boldface appeared in the list of 100 most significantly up- or downregulated genes in the PCDA analysis.

Acc#	Symbol	GeneName	Low		Mid		High		A
NM_022407	Aldh1a1	**Aldehyde dehydrogenase 1, A1**	0.80	*	1.54	***	2.28	***	***
M11794		**sim. to SMRT2 metallothionein II**	0.46		0.51		1.93	***	***
NM_013215	Afar	**Aflatoxin B1 aldehyde reductase**	0.59		0.97	**	1.85	***	***
NM_012695	Smp2a	**senescence marker protein 2A,**	0.77	*	1.20	***	1.76	***	***
J00750	Mt1a	**Metallothionein**	0.32		0.53	***	1.72	***	***
NM_016999	Cyp4b1	**Cytochrome P450, subfamily IVB, 1**	0.22		1.11	**	1.65	***	***
X89603	Mt3	**Metallothionein 3**	0.45		0.22		1.63	***	***
M31363	Sth2	**Sulfotransferase hydroxysteroid 2**	0.52	*	1.13	***	1.59	***	***
NM_031509	Gsta1	**Glutathione-S-transferase, alpha**	0.42	**	1.03	***	1.44	***	***
M13506	Udpgtr2	**UDP-glucuronosyltransferase**	0.42		0.79	**	1.22	***	***
NM_017000	Nqo1	**Diaphorase (NADH/NADPH)**	-0.08		0.54		1.22	**	***
NM_019286	Adh1	**Alcohol dehydrogenase I alpha**	0.29		0.21		1.01	*	**
D30035	Prdx1	**Peroxiredoxin 1**	0.55	**	0.51	**	0.87	**	***
NM_012844	Ephx1	**Epoxide hydrolase 1 (microsomal)**	0.29		0.23		0.81	*	**
M15327	Adh1	**Alcohol dehydrogenase**	-0.23		0.08		0.70	*	***
NM_031543	Cyp2e1	**Cytochrome P450, 2e1**	-0.04		0.32		0.64	**	***
NM_017305	Gclm	Glutamate-cysteine ligase, regulatory	0.29		0.42	*	0.62	**	**
NM_012709	Cyp2c12	**Cytochrome P450 15-beta**	0.17		0.15		0.60	**	**
NM_031000	Akr1a1	**Aldo-keto reductase family 1, A1**	0.28		0.42		0.59	**	*
NM_023025	Cyp2j4	Cyp2J4	0.01		0.27	**	0.54	*	**
J05181	Gclc	Glutamylcysteine gamma synthetase light chain	0.73		0.35	**	0.53	**	*
NM_012541	Cyp1a2	Cytochrome P450, 1a2	0.06		-0.20		0.48		**
NM_031731	Aldh3a2	Aldehyde dehydrogenase family 3, A2	0.18		-0.44		0.37		**
BG668294	Esd	Esterase D/formylglutathione hydrolase	0.39		0.44	**	0.35		**
NM_031154	Gstm3	Glutathione S-transferase, mu type 3	0.05		0.06		-0.43	**	**
L26267	Nfkb1	Nuclear factor kappa B p105 subunit	-0.29		-0.19		-0.48	**	*
NM_012661	Sts	Steroid sulfatase	-0.72	*	-0.28		-0.65	**	**
NM_019184	Cyp2c11	**Cytochrome P450, subfamily IIC**	0.17		-0.77		-1.48	**	**

Protein synthesis & proteolysis – The gene expression data suggest increased protein turnover, as ribosomal subunits and many subunits of the proteasome were significantly induced. Possibly, proteolysis and protein synthesis take place in different cells, or in different cellular compartments.

Table VI.2C. *Genes modulated by trichloroethylene, related to protein synthesis and proteolysis.*
The level of statistical significance (Student's T-tests) is indicated to the mean log(2) transformed fold changes, and listed in the last column for the ANOVA (**A**) (*: $p < 0.02$, **: $p < 0.01$, ***: $p<0.001$). Genes in boldface appeared in the list of 100 most significantly up- or downregulated genes in the PCDA analysis.

Acc#	Symbol	GeneName	Low	Mid	High	A
NM_022672	Rps14	**Ribosomal protein S14**	0.63*	0.56	0.98*	*
X52783	Psmb1	**Proteasome subunit, beta type 1**	0.57**	0.54**	0.81	**
U53512	Mrpl17	**Mit. ribosomal protein L17**	0.55*	0.58*	0.75**	**
BU671701		sim. to eukar. translation initiation factor 3 sub6 interacting prot	0.85	0.43	0.66	**
D50695	Psmc4	Proteasome 26S subunit, ATPase, 4	-0.30	0.37	0.55**	***
NM_017280	Psma3	Proteasome subunit, alpha type 3	0.35*	0.46**	0.52**	**
NM_031838	Rps2	Ribosomal protein S2	-0.04	0.33	0.47*	**
NM_017285	Psmb3	Proteasome subunit, beta type 3	0.10	0.42**	0.40***	***
NM_017282	Psma5	Proteasome subunit, alpha type 5	0.44**	0.22	0.38*	***
AF285103	Psmb7	Proteasome subunit, beta type, 7	-0.19	0.31	0.36	**
NM_031570	Rps7	Ribosomal protein S7	0.63**	0.29	0.33	**
NM_017281	Psma4	Proteasome subunit, alpha type 4	0.16	0.32*	0.31**	**
D50694	Psmc2	Proteasome 26S subunit, ATPase	-0.26	0.32	0.22	**
AF146518	Enpep	aminopeptidase A short variant	-0.36	0.04	-0.35**	**

Lipids, fatty acids and cholesterol metabolism
Gene expression changes indicated that the metabolism of lipids, fatty acids and of sterols including cholesterol was affected by TCE. The peroxisomal beta oxidation of fatty acids was influenced at the gene expression level, bu induction of a.o. Acat1, Cyp4A, Dci, Echs1, Ehhadh. These results corroborate reported TCE-induced peroxisomal beta oxidation (increased relative liver weights and palmitoyl CoA oxidation activity) [182]. Proliferation of peroxisomes (PP) may be a response to perturbations in lipid metabolism [188] and is putatively associated with carcinogenesis [189]. A comparison was made of gene expression changes in rat livers upon exposure to TCE and model peroxisome proliferators such as Wy14,643, Clofibrate, gemfbrozil [190], [27];[27,191](table VI.3.). Several studies with peroxisome proliferators were performed in mice, not included in present comparison [192], [193], [194]. The comparison indicated that TCE altered the expression of hepatic genes characteristic for the response induced by peroxisome proliferating agents. This characteristic set of genes included enzymes involved in the beta oxidation of fatty acids. In agreement, TCE also increased relative liver weight. Alternatively, this relative liver weight could have been increased by the marked induction of biotransformation enzymes.

Table VI.2D. *Genes modulated by trichloroethylene, related to fatty acids, cholesterol and lipids metabolism.*
The level of statistical significance (Student's T-tests) is indicated to the mean log(2) transformed fold changes, and listed in the last column for the ANOVA (**A**) (*: $p < 0.02$, **: $p < 0.01$, ***: $p < 0.001$). Genes in boldface appeared in the list of 100 most significantly up- or downregulated genes in the PCDA analysis.

Acc#	Symbol	GeneName	Low	Mid	High	A
NM_031561	Cd36	**Cd36 antigen**	0.35	1.21***	2.16***	***
D00729	Dci	**delta3, delta2-enoyl-CoA isomerase**	0.30	0.70	1.98***	**
NM_017306	Dci	**Dodecenoyl-coenzyme A delta isomerase**	0.73	0.56	1.87**	**
X55969	Apob	**apolipoprotein B**	0.93	0.92	1.07	*
X97831	Slc25	**Solute carrier family 25 (carnitine/acylcarnitine translocase)**	0.83**	0.40	0.91**	**
M57719	Cyp4A	**Cyp4a cytochrome P450 (IVA3)**	0.05	0.67**	0.91**	***
NM_130433	Acaa2	**acetyl-Coenzyme A acyltransferase 2 (mit.3-oxoacyl-CoA thiolase)**	0.49	0.21	0.87	*
K03249	Ehhadh	**Enoyl-CoA, hydratase/3-hydroxyacyl CoA dehydrogenase**	0.15	0.37	0.84***	**
NM_012930	Cpt2	**Carnitine palmitoyltransferase 2**	-0.08	0.24	0.67**	**
NM_031344	Fads2	**Delta-6 fatty acid desaturase**	-0.14	0.03	0.59**	***
D85189	Facl4	**Fatty acid Coenzyme A ligase, long chain 4**	0.30	0.33**	0.57***	***
X15958	Ech1	Enoyl CoA hydratase, short chain 1	0.44	0.21	0.47*	*
NM_017075	Acat1	Acetyl-Co A acetyltransferase 1, mitochondrial	0.27	0.40**	0.42**	*
NM_138502	Mgll	monoglyceride lipase	-0.27	0.30	0.40	**
NM_022389	Dhcr7	7-dehydrocholesterol reductase	-0.20	-0.05	0.27	***
AF080568	Pcyt2	Phosphate cytidylyltransferase 2, ethanolamine	-0.31**	0.06	-0.12	**
NM_013112	Apoa2	Apolipoprotein A-II	-0.42	-0.41***	-0.42	*
AJ245707	Hpcl2	2-hydroxyphytanoyl-CoA lyase	-0.33	-0.07	-0.45**	**
AF202887	Apoa5	**Apolipoprotein A-V**	-0.13	-0.89**	-0.78**	**
NM_017332	Fasn	**Fatty acid synthase**	-0.87*	-0.82**	-0.81**	**
NM_012737	Apoa4	**Apolipoprotein A-IV**	-0.46	-1.11**	-1.72***	***

Table VI.3. *Gene expression changes by TCE and. peroxisome proliferators*

A selection of genes is shown, of which the expression changed by **TCE** as well as by peroxisome proliferators, identified in studies by Cornwell *et al.* **(PP1),** Hamadeh *et al.*,**(PP2)** and McMillian *et al.*(**PP3**) .

Symbol	Gene Name	TCE	PP 1	PP 2	PP 3
Acs	acyl-CoA oxidase	+	+	+	+
Cyp4A3	cytochrome P450 4a3	+	+		+
Dci	δ3, δ2-enoyl CoA isomerase	+	+	+	+
Cpt2	carnitine palmitoyltransferase 2	+	+		+
Acat1	acyl-CoA acetyltransferase	+	+		+
Acaa	acetyl-CoA acyltransferase 1	+	+	+	+?
Ehhadh / Echp	peroxisomal bifunctional enzyme	+	+		
Ech1	enoyl CoA hydratase, short chain 1	+	+		+
Cd36	cd36 antigen (fatty acid binding)	+			+
Hsd11	hydroxysteroid dehydrogenase 11 beta	-		-	-
Apoa4	apolipoprotein A IV	-	-		
ApoC3	apolipoprotein C-III		-	-	
Fasn	fatty acid synthase	-		+	
Mgll	monoglyceride lipase	+	+		
Atp	div. ATPases	+	+		
Me1	malic enzyme 1	+	+		+
Pklr	pyruvate kinase	-	-		
Idh	isocitrate dehydrogenase	+	+		
-	(sim.to) Gadd45	+	+		
Ces	carboxylesterase (2)	+		+	+
Nqo1	NAD(P)H dehydrogenase, quinone 1	+		+	
Aldh1	aldehyde dehydrogenase 1	+		+	
Gsta1	GSH-S-transferase A	+		+	
G6pc	glucose-6-phosphatase	-		-	-
-	div. complement components	-		-	
Aqp...	div. aquaporins	+	+		-
Obp3	alpha 2u-globulin	-			
Mt...	metallothionein	+			-

+ : induction of gene expression ; -: reduction of gene expression

Table VI.4. *Gene expression changes by TCE vs. other compounds*
A selection of genes is shown of which the expression changed upon 28-day exposure to trichloroethylene **(T),** benzene **(B)** (Heijne *et al*, 2004), as well as by the model hepatotoxicants acetaminophen **(A)** (Heinloth *et al.*,2004), or bromobenzene **(BB)** (Heijne *et al*, 2004).

Symbol	Gene	Acc.#[1]	Acc.#[2]	BB	A	B	T
	biotransformation						
Ephx1	microsomal epoxide hydrolase	NM_012844	AA900551	+	+	+	+
Afar	Aflatoxin B1 aldehyde reductase	NM_013215	AA923966	+	-	+	+
Gsta	Glutathione-S-transferase alpha	NM_031509	AA818339	+	=/+	+	+
Aldh	Aldehyde dehydrogenases	div	div	+	=	+	+
Ugt	UDP-glucuronosyltransferases	div	div	+	=/+	+	+
Nqo1	NAD(P)H dehydrogenase, quinone 1	NM_017000	AA899180	+	+	+	+
Cyp2c12	Cytochrome P450 15-beta	NM_031572	AA818124	-	-	-	?
Cyp2e1	Cytochrome P450, 2e1	NM_031543	AA818896	-	-	+	+
	GSH metabolism and oxidative stress						
Gclc	Glutamylcysteine gamma synthetase	J05181	AA965220	+	?	+	+
Mt	div. metallothioneins	*div*	*div*	+	+	=	+
Timp1	TIMP1	-	AA957593	+	+	?	?
Prdx1	peroxiredoxin1	D30035	AA875245	+	+	=	+
Ho-1	heme oxygenase 1	NM_012580	AA874884	+	+	=	=
Ftl,Fth	ferritin light and heavy chain	*div*	*div*	+	=/+	=	=
	protein synthesis and proteolysis						
Rpn..	various ribosomal subunits	*div*	*div*	+	+	-	+
Psmb..	various proteasome subunits	*div*	*div*	+	=	=/-	+
	fatty acid metabolism						
div.	multiple genes	*div*	*div*	-	+/-	+	+
	glucose						
G6pc	Glucose-6-phosphatase	NM_013098	AA964628	-	-	-	-
G6pd	Glucose-6-phosphate dehydrogenase	NM_012942	AA923963	+	=	+	+
	diverse processes						
Orm	orosomucoid	J00696	AI029162	+	+?	=	=
Mug	alpha(1)-inhibitor 3	NM_017351	AA817965	-	-	=	-
Bhmt	betaine-homocysteine methyltransferase	NM_030850	AA901407	-	+	+	=
Actb	beta actin	NM_031144	AA859846	+	?	+	=
Hbb	hemoglobin beta	X05080	AA818084	+	nd	+/-	+
Hba	hemoglobin alpha	NM_013096	AA819784	+	-	+/-	=/+

#1: Accession number of DNA fragment on the microarrays in the B and TCE study
#2: Accession number of the cDNA on the microarrays in the BB and APAP studies
+ : induction; -: reduction; =: no (significant) change in gene expression levels
nd.: not determined; ?: equivocal results

Specificity of transcriptome changes related to hepatotoxicity

Previous transcriptomics studies analysed effects in livers of rats exposed to model hepatotoxicants like acetaminophen (APAP) [69] and, in our laboratory, bromobenzene (BB) [2]. These compounds induced many hepatic gene expression changes in parallel to hepatic necrosis and increases in the relative liver weight. While TCE also induced the relative liver weight, no histopathological or other markers provided indications of hepatotoxicity. Nonetheless, various changes in hepatic gene expression were found, and it is yet unclear whether these should be considered as reversible or irreversible and adverse effects. The TCE-induced changes in hepatic gene expression were therefore compared to those induced by the well-known hepatotoxic compounds BB and APAP. The effects of TCE were also compared to those of benzene, which is not a recognised hepatotoxicant, but, like TCE, increased relative liver weights [5](table VI.4).

TCE was found to modulate many genes in common with benzene. A few changes in gene expression were in common with the hepatotoxicants BB and APAP like induction of Ephx1, Nqo1, Aldh and reduction of G6pc. Some distinct effects were found between the model hepatotoxicants on the one hand, and benzene and TCE on the other. These include changes in genes related to fatty acid metabolism (eg. Ech1) and oxidative-stress response (HO-1). The induction of HO-1 and ferritins was found to be an early event in response to BB [3]. After 28 days of exposure to benzene and TCE, this effect could have vanished. Another study in our laboratory related changes in gene expressions to histopathological observations of necrosis [4]. In this way, induction of orosomucoid, actin beta, pervin and reduction of asialoglycoprotein 2 and lecithin-cholesterol acyltransferase (Lcat) were found to correlate to the degree of hepatic necrosis. These effects might be useful to differentiate between exposures to model hepatotoxicants and compounds like benzene and TCE, that did not induce histopathological effects in liver. TCE did not significantly alter the expression of any of those genes. Interestingly, a slight reduction of asialoglycoprotein 2 and Lcat was observed upon exposure to both B and TCE, though this decrease was not statistically significant. Possibly, this slight reduction is an early indication of adverse effects while larger decreases correlate with clear signs of necrosis. This hypothesis should be verified in further studies.

One of the earliest toxicogenomics studies measured hepatic gene expression for 148 genes after exposure to TCE, Benzo(a)pyrene or Cadmium chloride [94]. Differential gene expression was found in mouse livers 6 hours after a single dose of these compounds. The only genes reported to change (only) by TCE were Hsp25, Hsp86 and Cyp2a. Our study did not reveal expression changes for those genes. The comparison of these results with present study is difficult because of the large differences in the studies (eg. species, duration and doses of exposure and microarray platforms). This emphasises that it is important to standardise toxicogenomics studies to be able to relate results to each other.

Transcriptome changes and nephrotoxicity

Several hepatic gene expression changes were observed that might relate to mechanisms suggested to mediate TCE-induced nephrotoxicity and/or nephrocarcinogenesis. A possible mechanism involves GSH conjugation of TCE and its metabolites, probably important when the Cyp-dependent pathway becomes saturated at high doses of TCE [195]. The induction of Gclc and Gclm and corresponding increased GSH levels could therefore increase the GSH conjugation, thereby leading to formation of more nephrotoxic metabolites. Induction of Gsta expression might enhance this process. On the other hand, the induction of Cyp2e1 could

prevent that the Cyp-dependent pathway becomes saturated, attenuating the role of the GSH-dependent route.
This study revealed a 2.5-fold decrease in gene expression of alpha 2u-globulin in liver (figure VI.4). In male rats, alpha 2u-globulin accumulation in lysosomes causes hyalin protein droplet induction in the kidneys. This was associated to cell proliferation and raised as a possible explanation for chemical-induced carcinogenicity in male rats, though not in humans [196] [197], [198]. We suggest that the impaired hepatic gene expression of alpha 2u-globulin prevents accumulation of this protein in the kidneys, explaining the results of the kidney histopathology. Hyalin protein droplet induction was found in the controls and low dose animals and should be considered as a background effect, observed more often (Dr.B. Lina, personal communication). However, none of the animals treated with the mid and high dose of TCE showed hyalin droplet nephropathy. These findings may also explain why increase in renal alpha 2u-globulin concentration was not found upon exposure of male Fischer-344 rats to TCE [196].

Conclusions

In summary, TCE changed expression of genes in rat liver, after 28-day repeated exposure to 50 mg/kg/day. The expression of genes changed in a dose-dependent manner, both in the number and the magnitude of the changes. The only hepatic effects found with conventional toxicology were increase of relative liver weight and glutathione contents, observed after exposure to 500 mg/kg/day. Metabolomics analysis enabled to assess exposure levels of TCE, in a robust and specific analysis of urine. The TCE induced gene expression changes were predominantly related to GSH biosynthesis and biotransformation. TCE induced characteristic changes in hepatic gene expression in common with agents inducing peroxisome proliferation. Comparison of the effects of TCE with model hepatotoxicants that induce liver necrosis, showed a few genes modulated in common. On the other hand, a considerable overlap in effects of TCE and benzene was found. Both benzene and TCE increased the liver weights but did not induce histopathological changes in liver. Moreover, the liver plays a central role in the biotransformation and activation, ultimately leading to target organ toxicity (benzene:blood and TCE:kidney). Besides the overlap, a characteristic pattern of TCE-induced gene expression changes was observed, which enabled to further delineate the specificity of gene expression changes in liver after exposure to (potentially) hepatotoxic compounds.

Acknowledgements

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Chapter VII

Toxicogenomics analysis of joint effects of benzene and trichloroethylene mixtures in rats

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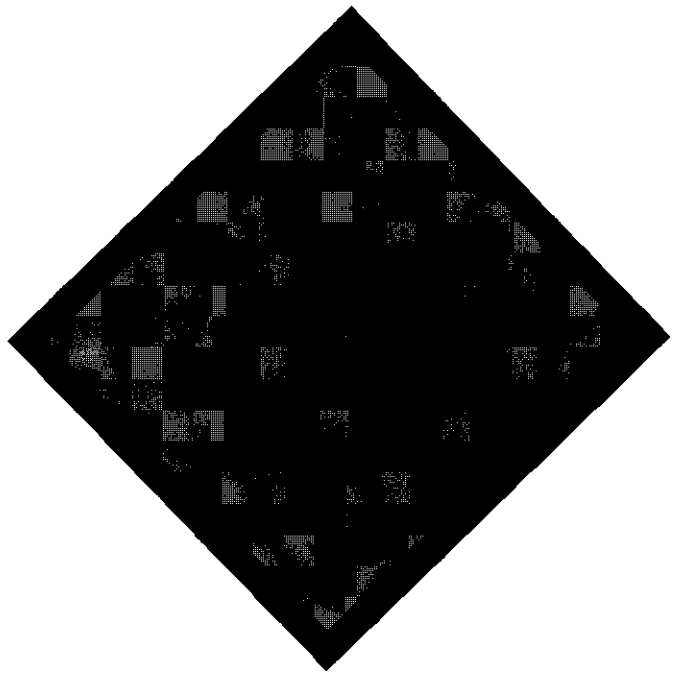

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Abstract

Benzene and trichloroethylene are widely used chemicals. They are toxic and (suspected) carcinogenic, and frequently occur (as mixtures) in soil and water. In the present study, joint effects of repeated oral exposure to mixtures of benzene and trichloroethylene were investigated in a stepwise approach.
First, joint effects of the mixtures were predicted from available data for the single compounds. Literature and range finding studies for subacute toxicity and hepatic gene expression profiling (transcriptomics) were used.
Subsequently, the response to benzene and trichloroethylene (mixtures) was tested in an *in vivo* subacute toxicity study in male F344 rats.
Benzene was hematotoxic while trichloroethylene affected the kidneys and liver. Most of the changes in routine toxicity end points caused by mixtures could be described by addition of effects of single compounds. The joint effects on relative lymphocyte and neutrophil counts, and, inexplicably, on red blood cell parameters were more pronounced than predicted.
Investigative toxicological methods were used to explain mechanisms of joint action. Transcriptomics analysis (DNA microarrays) identified changes related to bioactivation and detoxification, fatty acid metabolism and peroxisome proliferation. The data suggested involvement of trancriptional regulators like the electrophile response element (EpRE), the peroxisome-proliferation activated receptor (PPARα) and hepatocyte nuclear factor (Hnf3). The response of most genes to the mixtures could be estimated by combining the effects of the individual compounds.
Profile analysis with multivariate statistics exploited the large datasets to indicate that hepatic effects of trichloroethylene were enhanced in the presence of benzene.
In vitro exposed hepatocytes had increased biotransformation enzyme activity. According to *in silico* physiologically-based pharmacokinetic (PBPK) modelling, this enhanced bioactivation of trichloroethylene the metabolite presumably responsible for toxicity. This could explain how benzene enhanced the effects of trichloroethylene.
Present study demonstrates how investigative methods (transcriptomics, *in vitro* exposures and PBPK modelling) could complement conventional toxicology to assess and explain the effects of mixtures of (potentially) toxic substances.

VII.1 Introduction

The present study demonstrates how transcriptomics, *in vitro* exposure and physiologically-based pharmacokinetic (PBPK) modelling could complement conventional toxicological examinations to better assess and explain the effects of mixtures of the toxic chemicals B and TCE.

Benzene (B) and trichloroethylene (TCE) are widely used chemicals that frequently occur (as mixtures) in soil and water. They are toxic and (suspected) carcinogenic environmental pollutants and therefore of high concern. The toxic effects of benzene and trichloroethylene are exerted by secondary metabolites of the compounds, after metabolism in the liver. Benzene (metabolites) primarily induce bone marrow and blood toxicity. Chronic exposure to benzene may increase the risk of acute myelogenous leukemia [128]. In hepatic biotransformation of TCE, glutathione conjugates are formed that, after bioactivation, are toxic predominantly in the kidneys. Whether TCE is also nephrocarcinogenic is still a subject of debate [178];[185-186].The exact toxic effects of exposure to mixtures of B and TCE are yet unclear. It is expected that the liver plays an important role, and forms a putative site of interaction.

A stepwise approach was chosen to analyse mixture toxicity of B and TCE, as shown in figure VII.1. Initially, data were collected for the effects of the single compounds. Based on these data, the effects of mixtures were predicted. Subsequently, the hypotheses were tested in rats exposed to mixtures as described below. In the last step, new hypotheses were generated.

The research programme started with 28-day range finding studies with either B or TCE in F344 rats [5-6];[199]. The conventional subacute toxicity data for the single compounds, in combination with published literature, were used to predict (interaction) effects of exposure to mixtures [200]. The conventional toxicology was also integrated with DNA microarray based measurements of hepatic gene expression (transcriptomics). This enabled detailed analysis of molecular mechanisms in response to exposure to B and TCE. Thus, the application of transcriptomics should also enable better understanding of the molecular mechanisms of joint action of B and TCE. And, as thousands of parameters are measured with a large dynamic range, transcriptomics may provide better means to distinguish and (more) quantitatively assess joint action compared to the conventional toxicity endpoints.

Hypothesis generation and testing
Based on conventional toxicology, trichloroethylene was proposed to influence effects of benzene on the liver and spleen and hematological parameters. Benzene was suggested to influence effects of TCE on the liver and kidneys [200]. Transcriptomics analyses in the range finding studies provided indications for several transcriptional regulation mechanisms that could be important in the response to mixtures of B and TCE. These mechanisms were related to a.o. glutathione metabolism, fatty acid metabolism and peroxisomal proliferation. Therefore, hepatic gene expression profiles of selected rats were analysed after exposure to mixtures.

A putative mechanism of interaction upon exposure to mixtures that was investigated in more detail was the hepatic biotransformation of B and TCE. The mechanisms of hepatic

biotransformation of B and TCE share a number of enzymes, like the bioactivating enzyme cytochrome P450 2E (CYP2E) and conjugation enzymes like glutathione-S-transferases (GSTs) and UDP-glucuronyltransferases (UGTs) [207-208]. Therefore, toxicokinetic interactions may be expected after exposure to mixtures. These could determine toxicity in the liver, but also in kidney, bone marrow or other tissues. Both compounds are metabolised primarily by CYP2E, and were found to compete for the activation by CYP2E. It was reported that rats injected with mixtures of B and TCE showed reduced benzene metabolism as measured by conjugated phenol excretion [209]. This would imply that bioactivation of benzene is reduced after exposure to mixtures. Therefore, less than additive hematotoxic effects of benzene could be expected. Alternatively, if CYP activities would increase upon exposure to the mixtures, enhanced bioactivation and aggravated hematotoxic effects of benzene were considered more plausible.

Recently, Gonzalez-Jasso *et al.* reported the induction of CYP2E gene (and protein) expression in rats exposed to benzene [159]. In our range finding studies, TCE and B were found to increase relative liver weight, which could be associated with biotransformation enzyme induction [5-6];[199]. However, the total CYP protein levels did not change significantly. On the other hand, hepatic transcriptomics analyses revealed that both B and TCE induced gene expression of many biotransformation enzymes including the CYP2E isozyme. Thus, the induction of CYP2E and other biotransformation enzymes is likely to play a pivotal role in the toxicity by benzene, TCE and mixtures.

The present study with exposure of rats to mixtures aimed to shed more light on induction of biotransformation enzymes and other genes. In addition, F344 rat hepatocytes were exposed *in vitro* for specific measurements of CYP2E and GST activity. Moreover, literature-based PBPK modelling was applied to test the influence of enzyme induction. Especially the implications of benzene-induced oxidative metabolism of TCE were analysed in this *in silico* approach.

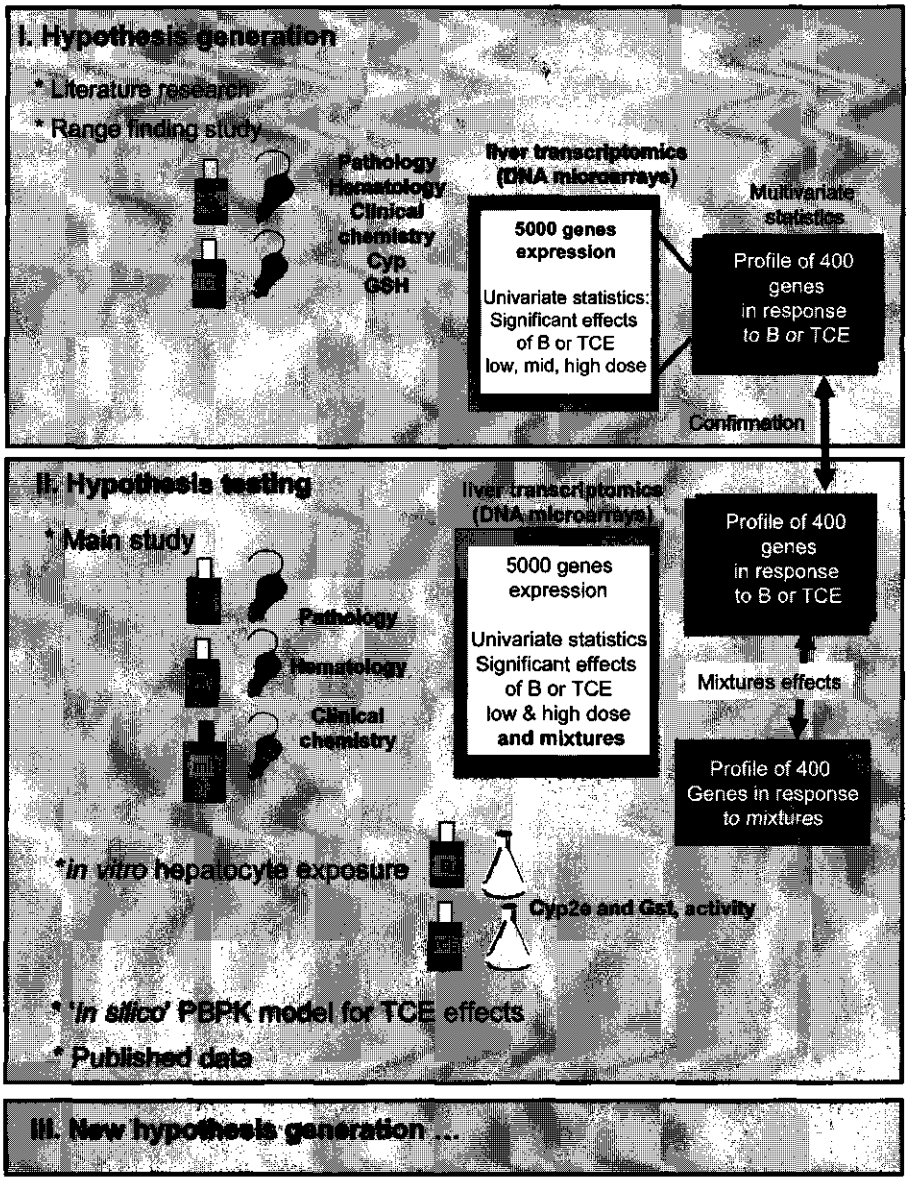

Figure VII.1 *Integrated approach for toxicological analysis of mixtures effects*
Hypotheses were generated using data about the single compounds, from literature and the range finding studies. Hypotheses were tested in the main study, where rats were exposed to benzene (B), trichloroethylene (TCE) and mixtures. Gene expression was analysed with univariate and with multivariate statistical methods. In addition, hepatocytes were exposed *in vitro*, and important biotransformation enzyme activities were measured (GST and CYP2E). Physiologically based pharmacokinetic (PBPK) modelling with data from literature were used to determine the implications of enzyme activity changes.

VII.2 Materials and methods

In vivo subacute toxicity study with (mixtures of) B and TCE

The toxicity study were performed as reported earlier by Jonker *et al.* [199]. Benzene (> 99% purity) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), TCE (> 99% purity) (Aldrich Chemical Co., Milwaukee, USA), and corn oil (Remia, Den Dolder, The Netherlands) were administered orally to male F344 rats (CDFF® (F344)/CrlBR) (Charles River Wiga GmbH, Sulzfeld, Germany). The welfare of the animals was maintained in accordance with the general principles of the European Communities (Directive 86/609/EEC) and Dutch legislation. Throughout the study, feed (RM3 from Special Diets Services, Witham, England) and tap water were freely available.

B and TCE were dissolved in corn oil and administered by daily oral gavage in a volume of 5 ml per kg body weight to groups of 5 rats. Fresh dosing solutions were prepared once and stored (at 2-10°C) in portions sufficient for one day. Prior to the corn oil dose, all animals received a similar volume of tap water (because tap water was the vehicle for another compound which was tested in this study though not included in present work) . The dose levels were based on previous range-finding studies [5-6]. TCE was given at 500 (Tl, low dose) or 1500 (Th, high dose) mg/kg body weight/day. B was given at a low dose (Bl) of 200 mg/kg/day, or at a high dose (Bh) of 600 (days 0-2) or 400 (from day 10) mg/kg/day. Four mixture groups received B plus TCE at combinations of these dose levels: BlTl, BhTl, BlTh or BhTh. Two control groups of 5 rats each (CO_1 and CO_2), were dosed with tap water and corn oil (5 ml/kg body weight of each) only. The dose volumes were adjusted twice per week for changes in body weight. The intended treatment period of 28 days was not achieved for several reasons. Initially, unexpectedly high toxicity of B necessitated discontinuation of treatment on day 3. Following a treatment-free period of 7 days, dosing was restarted on day 10 with reduced levels of B. Thereafter, it became increasingly difficult to administer the animals the second (corn oil) dose, several animals died from gavage trauma and treatment was terminated after the 14th/15th dose. The animals received their last dose on the day before sacrifice on days 23-24. Examinations and measurements to detect toxicity included daily clinical observations, body weight, food and water consumption, routine hematology and clinical chemistry, organ weights, and (histo)pathology (for details see Jonker *et al.* [199]. Statistical analyses were conducted by an initial F-test followed by t-tests. In addition, the results for the mixture groups were analysed for departures from effect addition (interaction), by comparing the data with an additivity surface (as described by Jonker *et al.* [199]. Animals showing evidence of gavage damage were excluded from the statistical analyses.

Liver RNA extraction

Liver samples were pulverised in liquid nitrogen before isolation of total RNA, using Trizol according to the manufacturer's protocol (Invitrogen, Breda, The Netherlands). RNA was further purified using the RNEasy purification kit, including a DNA digestion by RNAse-free DNAsel (Qiagen, Westburg B.V., Leusden, Netherlands). RNA was checked for purity and stability by gel electrophoresis and the concentration was calculated from the OD_{260} as determined spectrophotometrically.

DNA microarray experiment design, quality criteria and data (pre)processing

A referenced design was used for microarray hybridisation, to account for dye-dependent effects. RNAs of individual rat livers were labelled with fluorescent Cy5, while reference material, previously obtained from RNA of the livers of control rats [5], was labelled with Cy3. The rat oligonucleotide microarrays used in this study were produced in collaboration with the UMC Utrecht genomics lab (Utrecht, The Netherlands), as described before [5]. Technical replicate hybridisations were performed to obtain, per animal, two microarrays, meeting stringent quality criteria described before [5]. After image analysis, the local background intensity value was subtracted from the signal intensity for each spot. The minimal signal intensity was set to 1.5 x background for the two channels. Flagged spots and controls were excluded from further interpretation. Genes for which less than 70 % of the microarrays delivered a significant signal were excluded from further interpretation. Ratios of the background-corrected intensities of tester over reference were calculated for each slide and, normalised using the lowess normalization algorithm [103] in the SAS statistical program. Data were log-transformed, with base 2, and excluded values were replaced by 0. Two-sided Student's T-tests were performed to assess the significance of the differences in gene expression in the test groups

compared to the controls. Changes were considered statistically significant if the p-value did not exceed 0.005.

Transcriptomics - Confirmation of individual gene expression changes by B and TCE
The response in the main study, of the genes found to change in the range finding studies, was assessed in samples exposed to either B or TCE. This was achieved by calculation of the average difference between relative gene expression levels (log(2)) in the samples treated with a high dose of benzene or TCE, and the controls. A (arbitrary) minimum increase or decrease of 0.3 on the log(2) scale (1.2-fold) was required for a gene to be designated as *confirmed* to change in accordance with previous findings.

Transcriptomics - Multivariate statistical analysis of expression profiles with PLS models
Partial least squares (PLS) regression analysis was carried out in Matlab (Version 6.5, The MathWorks Inc., Natick, MA, USA) using the PLS toolbox (Version 3.0, Eigenvector Research Inc., Manson, WA, USA). PLS models were generated to fit data from range finding studies. Log(2) transformed gene expression data were used as independent variables and exposure levels of TCE and B, respectively, as variables to be predicted. Full leave-one-out cross-validation was performed. Comparison of the predictions with the actual exposure levels, enabled to calculate the coefficient of correlation R^2 as a measure of accuracy of the model. We required an arbitrary threshold of $R^2 > 0.7$ to consider a model suitable for further analysis. Subsequently, the obtained PLS models were used to predict dose-equivalent levels based on gene expression data from the mixture study. Comparison of the predictions with the actual exposure dose levels enabled to calculate the coefficient of correlation R^2 as a measure of the accuracy of the predictions, and possibly as a measure to assess the occurrence of interaction effects, if gene expression changes would deviate from the expected effects.

"In vitro" exposure of rat hepatocyte cultures and measurement of CYP2E and GST activity
Primary rat hepatocytes were isolated from the liver of a male F344 rat, according to Seglen [201] and [202]. Collagen gel sandwich cultures of isolated rat hepatocytes were prepared, by coating the bottom of 75 cm^2 culture flasks with collagen. Freshly isolated primary hepatocytes were plated at a density of approximately 7×10^4 cells/cm^2 in Dulbecco's Modified Eagles Medium with 4.5 mg/ml glucose and L-glutamin supplemented with foetal calf serum (10% v/v), insulin (4 µg/ml), glucagon (0.007 µg/ml), hydrocortisone hemisuccinate (7.5 µg/ml), and gentamycin. Hepatocytes were attached to the collagen gels in 95% air and 5% CO2 at 37°C for 4 hours. Culture medium was removed and cells were covered with collagen for a period of 45 - 60 minutes. Serum-free culture medium (12 ml) was added, and used throughout the experiment. Cells were exposed to benzene or TCE dissolved in DMSO (final concentration DMSO 0.1%). Fresh medium containing the test compounds was added daily. Hepatocytes were exposed to a concentration range of B or TCE of 0, 10, 100, 500, 1000, 5000 and 10000 micromoles per liter. During the exposure, the flasks were sealed with parafilm to limit evaporation. After 4 days, cells were harvested by collagenase digestion and S9 fractions were prepared for enzyme activity measurements. Viability of the cells was assessed by measuring LDH levels in the medium. CYP2E activity was determined through the formation of 6-hydroxychlorzoxazone from chlorzoxazone, assayed by HLPC UV analysis [203]. GST enzyme activity was determined in S9 fractions according to Habig et al. [82]. Protein concentrations in S9 mixtures were determined according to Bradford [82].

"In silico "physiologically based pharmacokinetic modelling
A physiologically based pharmacokinetic (PBPK) model developed by Clewell *et al* [204] was used to investigate the effect of increased CYP2E activity on TCE metabolites like trichloroacetic acid (TCA). The rat PBPK model was run to determine effects of an oral dose of 2000 mg/kg/day of TCE using all parameters given in the original publication. A second run was performed to simulate the interaction with benzene, where the oxidative metabolism step (Vmax-ox) was increased by the factor found after treating hepatocytes with benzene.

VII.3 Results

This study assessed the effects of (mixtures of) B and TCE, with conventional toxicological methods, hepatic gene expression profiling, *in vitro* exposures of hepatocytes and PBPK toxicokinetics modelling. For that purpose, F344 rats were exposed to benzene, trichloroethylene or mixtures. Two dose levels were chosen that previously showed effects with conventional toxicological methods and/or transcriptomics measurements [5-6].

In vivo subacute toxicity studies
The results obtained for animals given B or TCE alone or as a mixture generally confirmed the organ-specific effects observed in the range-finding studies with the single compounds. A complete description is reported by Jonker *et al.* [199]. The main findings are summarized below.
Growth was not affected by the low dose of B or TCE. The initial high dose of B (600 mg/kg) caused marked growth retardaton or even weight loss. Growth of animals given the reduced high dose of B (400 mg/kg), the high dose of TCE, or the mixtures was slightly to moderatly retarded (see Table VII.1). The rats with reduced growth also showed decreased food consumption.

Hematology - TCE did not affect red blood cell, white blood cell or coagulation parameters. Both dose levels of B reduced, to about the same extent, total white blood cell count and the absolute numbers of lymphocytes and neutrophils. Similar changes in total or differential white blood cell counts were observed in the four mixture groups, most markedly in those given the high dose of B. In addition, the percentage of lymphocytes was decreased and that of neutrophils increased in the BhTh-group.

Clinical chemistry - Animals given a high dose of B showed a decrease in plasma creatinine and urea. Animals given TCE at the high dose showed an increase in plasma albumin and a decrease in total cholesterol. Rats exposed to mixtures had increased plasma albumin and decreases in bilirubin, creatinine, urea, and cholesterol.

Pathology - Spleen weights decreased dose-dependently in animals given B (Table VII.1). Animals given the low (but not the high) dose of B showed increased weights of the liver and kidneys. TCE increased weights of the liver and kidneys at both dose levels. Animals given the mixtures showed increased liver and kidney weights and decreased spleen weight. Macroscopic examination at necropsy revealed no compound-related changes. Histopathological examination (no table presented) revealed karyomegaly, especially in the inner cortical region, in the kidneys of animals given TCE alone or in combination with B. The incidence and/or severity increased with dose. Compared with exposure to TCE alone, co-exposure to B tended to aggravate the karyomegaly. Animals given B alone showed no compound-related histopathological changes.

Analysis of the mixture data for departure from additivity revealed statistically significant interactions for some of the conventional endpoints, namely: percentages of lymphocytes and neutrophils, red blood cell count, hemoglobin and packed cell volume. The changes in these endpoints (decreases, except for neutrophils which were increased) were more pronounced than expected under the assumption of additivity. The interaction observed for

the red blood cell values was unexpected because neither B nor TCE affected red blood cell values when given alone.

Table VII.1. *Body and organ weights*
Terminal body weights, relative liver, kidneys and spleen weights of the rats treated with benzene, TCE, or mixtures.

Group	B	TCE	Body	Rel. liver	Rel. kidney	Rel. spleen
	(mg/kg/day)		(g)	(g/kg BW)	(g/kg BW)	(g/kg BW)
CO_I	0	0	200.4 ± 3.7	39.9 ± 1.4	7.34 ± 0.10	2.59 ± 0.04
CO_II	0	0	199.0 ± 8.5	39.6 ± 1.2	7.28 ± 0.20	2.45 ± 0.05
BI	200	0	201.1 ± 4.4	45.4 ± 0.7**	8.18 ± 0.11*	2.30 ± 0.09
Bh	600 (400)	0	184.4 ± 8.8*	41.5 ± 0.4	7.65 ± 0.07	2.15 ± 0.03*
TI	0	500	202.3 ± 1.0	47.1 ± 2.0**	8.42 ± 0.14*	2.84 ± 0.53
Th	0	1500	183.8 ± 4.0	50.0 ± 1.8**	8.44 ± 0.23**	2.39 ± 0.06
BI TI	200	500	183.8 ± 5.6*	45.4 ± 0.7**	7.99 ± 0.15*	2.20 ± 0.04*
Bh TI	600 (400)	500	169.1 ± 5.5**	46.6 ± 0.9**	8.28 ± 0.22**	1.98 ± 0.12**
BI Th	200	1500	167.5 ± 7.5**	49.2 ± 1.1**	8.19 ± 0.13**	2.24 ± 0.04
Bh Th	600 (400)	1500	162.7 ± 7.4**	51.8 ± 1.4**	8.55 ± 0.20**	2.17 ± 0.05*

n = 5 or all groups except TI:n =2 and BI: n =4
Significance: One-way ANOVA + Dunnetts tests: *: p< 0.05; **: p< 0.01

Hepatic gene expression profiling (transcriptomics)
Gene expression was determined in livers of selected rats exposed to B, TCE or mixtures, using oligonucleotide DNA microarrays. The intention of this study was to highlight possible molecular mechanisms at the hepatic gene expression level for further studies. Because of this, and the availability of data from previous studies [5-6], the analysis was limited to two technical replicate measurements of two animals per group. Further, conclusive transcriptomics experiments would require a larger design, with replicated measurements of at least 3-5 rats per group. The average gene expression levels in two rats per treatment group were compared with controls, selected from both control groups, CO_I and CO_II. The gene expression changes were analysed with two separate approaches. Univariate statistics were applied to analyse changes in expression of *individual genes*, while multivariate statistics were used to analyse *profiles* of hepatic gene expression, in response to the different treatments.

Statistical significance of the changes was tested with univariate tests for 2437 genes left after quality filtering, and the number of changes was determined at two statistical confidence levels (Table VII.2 and figure VII.2). Both B and TCE altered expression of several genes at low doses, while higher doses affected more genes. Mixtures of B and TCE had the most pronounced effect on gene expression when TCE was included at high dose levels.

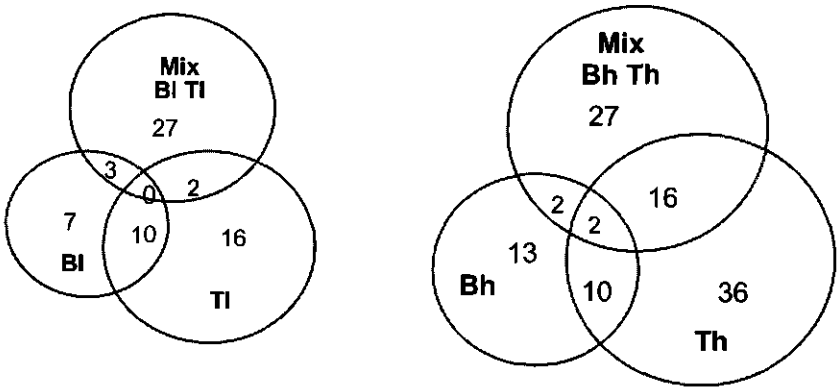

Figure VII.2 *Numbers of gene expression changes*
Venn diagrams of numbers of genes of which expression significantly ($p<0.005$) changed in the different treatment groups. B:benzene; T:trichloroethylene, l: low dose, h: high dose

Table VII.2. *Numbers of genes differentially expressed after treatment*
Two-sided Student's T-tests were performed to identify the number of changes

confidence:	Bl	Bh	Tl	Th	Bl Tl	Bl Th	Bh Tl	Bh Th
$p < 0.001$	1	5	5	13	10	14	4	16
$p < 0.005$	10	27	18	54	32	60	15	47

Benzene dose-dependently induced gene expression of Aflatoxin B1 aldehyde reductase (Afar), ribosomal subunits and cystathionine beta synthase (> two-fold and $p < 0.005$ at high dose). Tyrosine aminotransferase (Tat), aminolevulinic acid synthase 1 (Alas1) and the amino acid transporter A2 (solute carrier, Slc38a2) were dose-dependently reduced. TCE altered expression of more genes than benzene, and most pronouncedly induced were Afar, Aldh1, dodecenoyl-coenzyme A delta isomerase (Dci), cystathionine beta synthase, Gstm, and ferritin. Markedly reduced were cadherin 17 (Cdh17), carnitine palmitoyltransferase 1, ornithine aminotransferase (Oat), tryptophan 2,3-dioxygenase, hydroxyacid oxidase (Hao3) and the Abcc9 transporter.

The confidence in the observed gene expression changes in the main study was increased by comparison with previously observed changes in the range finding studies with benzene [5] and TCE [6] , as described in the methods section. Confirmation was assessed for the 400 genes most significantly affected in the range finding studies, by either B or TCE. The two sets of 400 genes contained 110 genes in common, affected by benzene as well as TCE treatment. For benzene, 33 genes out of the 400 were confirmed to change in the main study, after treatment with the high dose of benzene (17 up and 16 down). Most of these confirmed changes were slight (< 2-fold), and only two changes were established with statistical significance. These were the 2-fold induction of the "Myeloid differentiation primary response gene 116" (Myd116) and the 2.2-fold decrease of the "amino acid transporter A2" (solute carrier Slc38a2). Myd116 displayed increased expression during progression of transformation and in response to DNA damaging agents [205]. Slc38a2 is upregulated by a variety of hormones, growth factors, mitogens and altered levels of intracellular amino acids.

The expression in the skeletal muscle and liver is regulated by glucagon and insulin, for instance in association with diabetes [206].
For TCE, 106 out of the 400 genes were confirmed to change in the main study, after treatment with with the high dose of TCE (57 up and 49 down). Many genes for which the changes were not confirmed, were found to exhibit a large interanimal variation in response, for example metallothioneins, orosomucoid, hemoglobin, Hnf4 and apolipoprotein B. The marked induction by TCE of many ribosome (and proteasome) subunits in the range finding study was not confirmed in the main study. Out of the 106 confirmed genes, 39 were statistically significantly ($p < 0.01$) different from controls after treatment with TCE. A few of those were modulated in a similar manner by a high dose of benzene (Afar, ferritin, complement component 9, Hsp8, Tat, syndecan and Slc38a2).

Mixtures of B and TCE (especially at high dose) modulated genes that were also modulated by benzene and/or TCE only (figure VII.2). At high dose levels, around one third of the TCE-modulated genes was also changed by the mixtures. The overlap with benzene-induced changes was minimal. The genes most pronouncedly induced by TCE were also markedly induced by the mixtures, like the biotransformation enzymes Afar, Aldh1 and the enzymes Cd36 and Dci, related to beta oxidation of fatty acids. Other genes significantly induced by the mixtures include epoxide hydrolases, GSH synthase, two DNA fragments representing a thyroid hormone responsive protein (NM_012703 and K01934), and the "kidney-specific protein" (AF062389). The mixtures significantly reduced expression of Cdh17, Oat, Hao3, alpha albumin, Cyp2c12, G6pc and transporters Slc38a2 and Abcc9. The oxidative stress responsive ferritin was induced by both TCE and the mixtures. Expression levels of genes with significant induction or reduction by TCE that were also modulated by the mixture at high dose levels, were listed in table VII.3 a and b. The genes with most pronouncedly changed expression were also displayed in figure VII.3.

To assess the mode of joint action to change gene expression, we acquired the test hypothesis that the effects of B and TCE at the gene expression level could be summed to roughly predict the effect of mixtures. For simplicity, this hypothesis assumed that unlimited induction of expression of genes was possible, and that other steps in the mechanisms of action were not of influence (e.g. saturation of molecular receptors, transcription factors etc.) at the dose levels studied. Also, the fact that gene expression changes were expressed as log(2) ratios (test sample / reference) was neglected. Without aiming to exactly quantify the effects of the mixtures, expected gene expression levels were predicted by addition of the effects observed in samples exposed to either B or TCE at high doses. These predicted levels for the mixtures were compared to the actual effects measured upon exposure to a mixture of B and TCE at high doses. For the majority of the genes, the response to mixtures was similar to the response to TCE, regardless of the presence of benzene. However, for several genes the effect of TCE was enhanced by benzene, as expected based on the effects of benzene only on these genes. These included Afar, G6pc, Cdh17 and Oat. For a few genes, the effects of the mixtures deviated from expectations (departing from additivity). In the mixtures group, the enzyme Cd36 was clearly induced more than expected. Genes that were induced less than expected included ferritin, Gstm and ribosomal protein L15, Hsp8, Tat, complement component 9 and Slc38a2.

Table VII.3 a *Hepatic expression levels of genes induced significantly by TCE*

TCE-induced hepatic gene expression levels. Only genes that confirmed previous results of the range finding studies are shown. Average log(2) gene expression changes, corrected for expression levels in controls. Values are shown for TCE at low or high dose, Benzene (B) at high dose and the mixture at high doses (BhTh). Genes in bold are also displayed with histograms in figure VII.4.
Statistical significance: Student's t-test ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$.

Sym.	ACC	GeneName	TCE low	TCE high	B high	mix BhTh
Afar	**NM_013215**	**Aflatoxin B1 aldehyde reductase**	**0.54****	**2.00****	**1.14*****	**2.31*****
Adh1	**NM_019286**	**Alcohol dehydrogenase (class I), alpha**	**0.96***	**1.60****	**-0.31**	**1.26****
Dci	**NM_017306**	**Dodecenoyl-coenzyme A delta isomerase**	**0.51**	**1.45****	**0.22**	**1.51****
Dci	D00729	delta3, delta2-enoyl-CoA isomerase	-	1.41**	0.26	1.60***
Adh1	M15327	Alcohol dehydrogenase (class I), alpha	0.92*	1.41**	-0.25	1.23*
Aldh1a1	**NM_022407**	**Aldehyde dehydrogenase 1, A1**	**0.18**	**1.29*****	**0.30**	**1.65*****
Ftl	NM_022500	Ferritin light chain 1	0.67	1.18**	0.89**	1.01**
Gst	NM_017014	Glutathione-S-transferase, mu type 2	0.46	1.06**	0.63*	0.90*
Ephx1	**NM_012844**	**Epoxide hydrolase (microsomal)**	**0.25**	**0.90****	**0.29**	**0.92****
Cd36	**NM_031561**	**Cd36 antigen**	**0.23**	**0.90****	**-0.31**	**1.67****
Cth	NM_017074	CTL target antigen	0.20	0.85***	0.36*	0.48*
Ces2	AB010632	Carboxylesterase 2 (intestine, liver)	-	0.82**	0.42	0.81*
Me1	NM_012600	Malic enzyme 1, soluble	-0.34	0.81***	0.19	0.80*
Rpn	X78167	Ribosomal protein L15	-	0.76**	0.70**	0.85**
Cyp4b	NM_016999	Cytochrome P450, subfamily IVB, 1	0.02	0.62*	-0.30	0.77*
Cyp2j4	NM_023025	CYP2J4	0.32	0.58*	0.12	0.81***
Adh7	X98746	Alcohol dehydrogenase 7 (class IV)	0.40	0.57*	0.03	0.50*
Gsta	NM_031509	GSTa, Glutathione-S-transferase, alpha	0.13	0.49*	0.23	0.75**

Table VII.3 b *Hepatic expression levels of genes reduced significantly by TCE*

TCE-reduced hepatic gene expression levels. Only genes that confirmed previous results of the range finding studies are shown Average log(2) gene expression changes, corrected for expression levels in controls. Values are shown for TCE at low or high dose, Benzene (B) at high dose and the mixture at high doses (BhTh). Genes in bold are also displayed with histograms in figure VII.4. Statistical significance: Student's t-test ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$.

Sym.	ACC	GeneName	TCE low	TCE high	B high	mix BhTh
Col3a1	X70369	Collagen, type III, alpha 1	-0.14	-0.51**	-0.24*	-0.59*
Hnf3	NM_017077	Hepatocyte nuclear factor 3 gamma	-0.43*	-0.56**	-0.27*	-0.52*
Sdc2	NM_013082	Syndecan 2	-0.20	-0.62**	-0.40**	-0.59**
Kynu	U68168	Kynureninase (L-kynurenine hydrolase)	-0.31	-0.66*	-0.29*	-0.59**
Fgb	M35602	Fibrinogen, B beta polypeptide	-0.13	-0.66**	-0.36	-0.49*
Sult1c2	AJ238392	Sulfotransferase family, cytosolic, 1C, 2	-0.16	-0.67**	-0.28	-0.82**
	X76456	**alpha albumin**	**-0.35**	**-0.68****	**-0.35**	**-1.03*****
G6pc	**NM_013098**	**Glucose-6-phosphatase**	**-0.77****	**-0.71***	**-0.51***	**-1.49*****
Crym	Y17328	Crystallin, mu	0.05	-0.74***	-0.43*	-0.75**
Ptp	X84004	Protein tyrosine phosphatase	-0.48	-0.77*	-0.95	-1.50**
Hsp8	NM_024351	Heat shock cognate protein 70	-0.51*	-0.80***	-0.82***	-0.46**
Inhbe	NM_031815	Activin beta E	-0.26	-0.93**	-0.09	-0.66*
Slc38a2	AF249673	Amino acid transporter system A2	-0.68	-0.96**	-1.14**	-0.69**
Abcc9	AF019628	ATP-binding cassette, sub-family C (CFTR/MRP) 9	-0.45	-1.00**	-0.28	-1.00**
Hoa3	NM_032082	Hydroxyacid oxidase (glycolate oxidase) 3	-0.47*	-1.05**	-0.63*	-1.23***
Cyp2c12	NM_031572	Cytochrom P450 2c12 (15-beta)	-0.58	-1.25**	-0.65	-1.50**
Oat	**NM_022521**	**Ornithine aminotransferase**	**-0.34**	**-1.31****	**-0.43**	**-1.63****
Cdh17	**X78997**	**Cadherin 17**	**-0.83***	**-1.62****	**-0.43**	**-2.20****

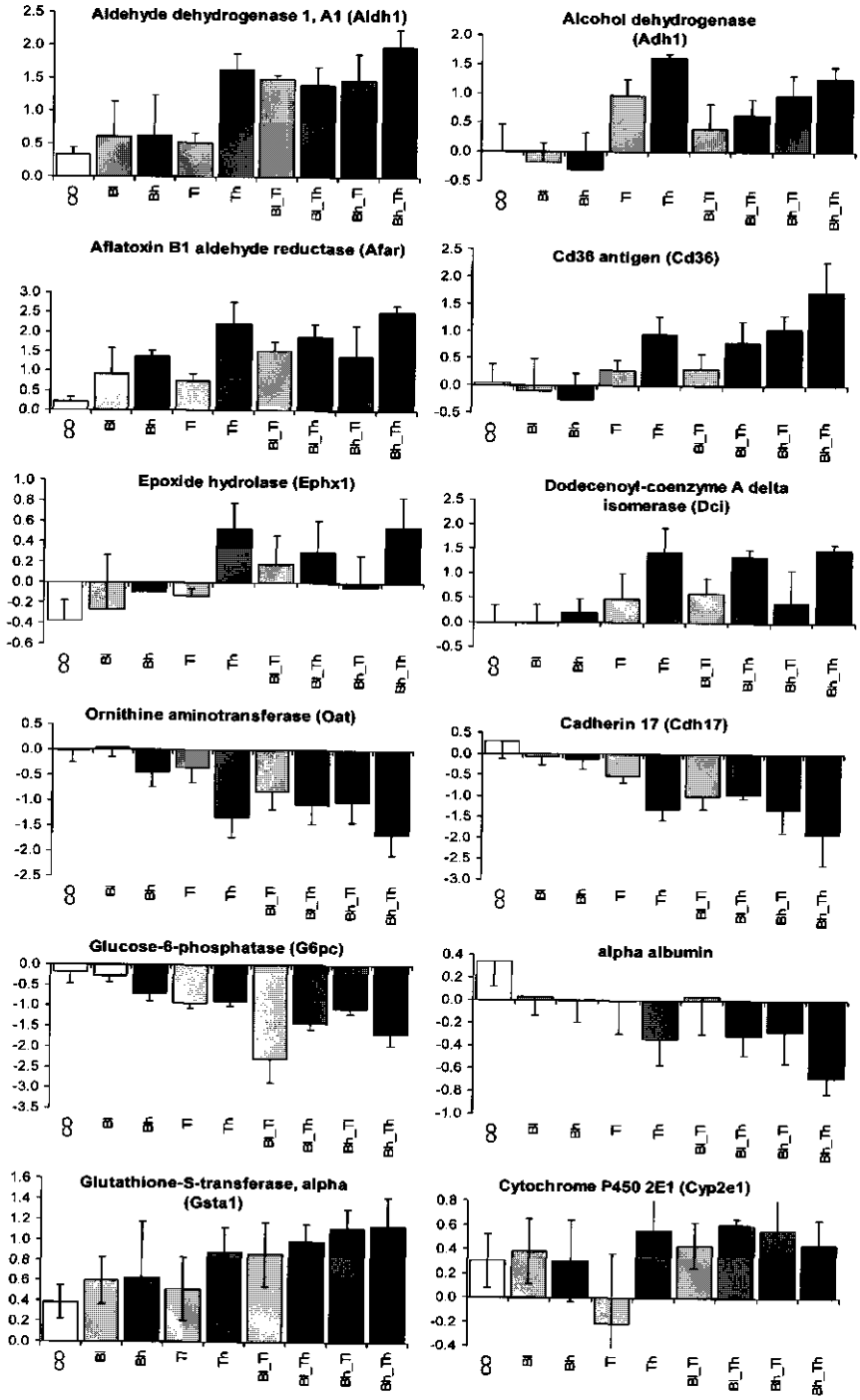
Aldehyde dehydrogenase 1, A1 (Aldh1)
Alcohol dehydrogenase (Adh1)
Aflatoxin B1 aldehyde reductase (Afar)
Cd36 antigen (Cd36)
Epoxide hydrolase (Ephx1)
Dodecenoyl-coenzyme A delta isomerase (Dci)
Ornithine aminotransferase (Oat)
Cadherin 17 (Cdh17)
Glucose-6-phosphatase (G6pc)
alpha albumin
Glutathione-S-transferase, alpha (Gsta1)
Cytochrome P450 2E1 (Cyp2e1)

Figure VII.3 (*previous page*)
The histograms display expression levels of genes significantly ($p<0.005$) modulated by trichloroethylene and modulated by mixtures of benzene and trichloroethylene. Bars represent mean log(2) expression levels of duplicate measurements of two rats per group (relative to reference), with error bars representing the standard deviations. In addition, gene expression changes in GSTa and CYP2E, important biotransformation enzymes for both compounds were displayed. Gsta1 gene expression was induced by the mixtures ($p = 0.006$ at BhTh, $p < 0.005$ at BlTh and BhTl). For CYP2E1, gene expression changes could not be established with statistically significance.

Multivariate statistical analysis of gene expression profiles
The multivariate statistical technique PLS (partial least squares) was used to assess the relation between exposure and hepatic gene expression profiles. Profiles measured in the range finding studies were used to construct PLS models that calculated "predicted exposure levels" for B and TCE. Subsequently, these predictions were correlated to the actual exposure levels. The robustness of the models was determined by leave-one-out cross-validation, and the accuracy of the models was shown by a coefficient of correlation, R^2, of 0.79 for benzene, and 0.85 for TCE. Based on these coefficients, both models were considered appropriate for subsequent analysis of hepatic gene expression in the main study.

The B and TCE PLS models were tested with samples from the main study (see methods section). Valid models should be able to relate gene expression profiles to exposure levels also in the main study. The coefficient of correlation between the predicted and actual exposure levels provided an indication for the validity of the model. As an arbitrary criterion, an R^2 of 0.7 or larger was chosen to consider a model valid. The TCE model was tested with samples from the main study (Figure VII.4a), and the predictions were in agreement with the actual exposure levels of TCE, as demonstrated by an R^2 of 0.88. This model was considered suitable for analysis of effects of mixtures. The same testing with the benzene model (figure VII.4b) showed that this model was not suitable to relate benzene-induced gene expression changes to the actual exposure levels of benzene in the main study, as demonstrated by an R^2 of 0.01. Therefore, no further attempt was made to assess the effects of the mixtures with this model.

The effects of mixtures of B and TCE on hepatic gene expression profiles were assessed with the PLS model for TCE. The gene expression changes were used to predict "exposure equivalents". These (on the y-axis) were correlated to the actual exposure levels of TCE (in the mixtures) (on the x-axis) (figure VII.5). The model calculated that the effects of mixtures were larger than the effects of TCE only. The analysis indicated that a mixture of low doses of TCE with B (Tl Bl and Tl Bh) affected gene expression more than low doses of TCE only. When the mixtures contained high levels of TCE, the effect of B in the mixtures got more pronounced. The low dose of B in the mixtures increased the predicted exposure equivalents by around 25%, while the high dose of B in the mixtures increased the effects by around 50%, as compared to high doses of TCE only. In conclusion, when a mixture of B and TCE was given, the TCE-induced effects on gene expression in rat liver were enhanced.

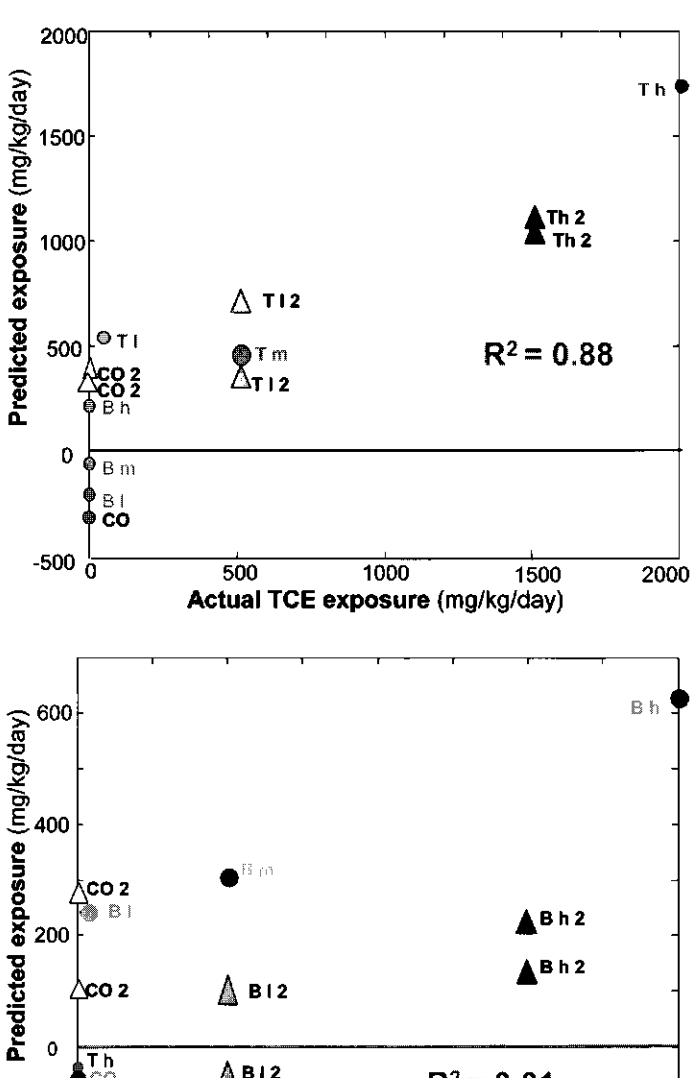

Figure VII.4 a, b *Testing of PLS models to relate exposure to gene expression*

The models based on the range finding study related the actual exposure to the gene expression levels in the main study (which were used to calculate "predicted exposure").
Upper panel a) Trichloroethylene: R^2 of 0.88 demonstrated that the TCE model could be used for further analysis of effects of mixtures.
Lower panel b) Benzene: R^2 of 0.01 demonstrated that the model was not suitable for further analysis Symbols: Range finding study: circles, Main study: triangles; CO: control; B: benzene; T:trichloroethylene; l:low, m:mid; h:high dose levels.

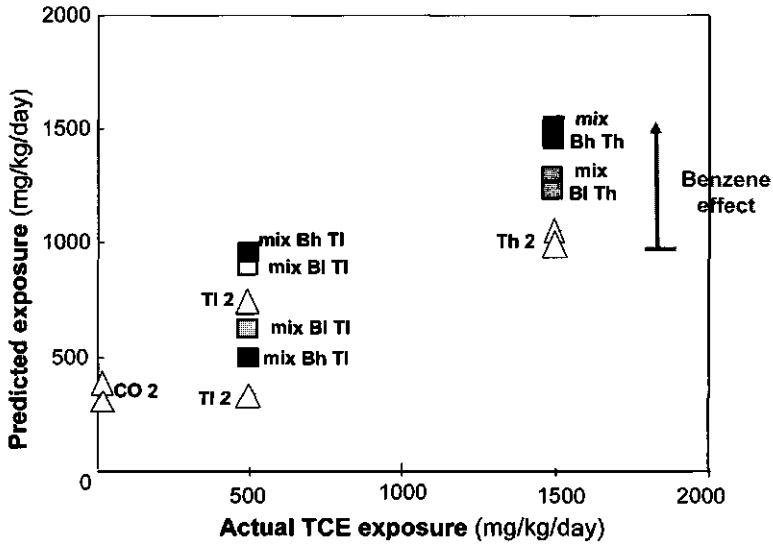

Figure VII.5 *Assessment of mixtures effects with PLS model for TCE*

The PLS model based on the effects of TCE in the range finding study could relate the actual exposure to the gene expression levels in the main study (used to calculate "predicted exposure"). The effects in samples treated with mixtures were larger compared to effects of TCE alone, especially at high doses. CO: control; B: benzene; T:trichloroethylene; l:low, m:mid; h:high dose

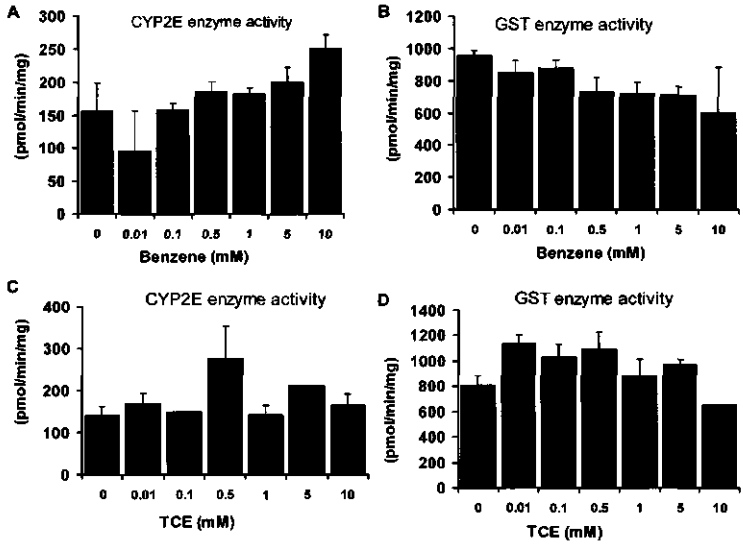

Figure VII.6 a,b *CYP2E and GST enzyme activity after benzene exposure,*

Figure VII.6 c,d *CYP2E and GST enzyme activity after trichloroethylene exposure*
Activity of cytochrome P450 2e (CYP2E) or glutathione S-transferase (GST) was measured after a four day exposure of hepatocytes in vitro to benzene or trichloroethylene
Data represent mean ± sd (n=3)

In vitro exposure of hepatocytes

CYP2E is the most relevant enzyme for bioactivation of both B and TCE in liver (hepatocytes). GSTs conjugate (reactive) metabolites of both compounds to glutathione. Previous experiments identified changes in Cyp2e and Gst gene expression, and therefore, activities were analysed in detail after 4 days of exposure of hepatocytes to various concentrations of the compounds *in vitro*. Activities were determined after four days of exposure of the cells to concentrations that did not induce cytotoxicity. Benzene dose-dependently elevated the levels of CYP2E activity to a maximum of 1.6 fold induction, and slightly decreased levels of GST activity. TCE increased GST activity, especially within the 0.01 - 0.5 mM concentration range. At 10 mM, GST activity decreased while cytotoxicity (based on LDH release) was not overt. A dose-related effect of TCE on CYP2E activity was not found. (figure VII.6)

In silico physiologically based pharmacokinetic modelling

The effect on TCE metabolism of CYP2E induction due to benzene in the mixture was analysed with a PBPK model developed by Clewell *et al.* [204] (methods section). The rat PBPK model was used to determine effects of an oral dose of 2000 mg/kg/day of TCE using all parameters given in the original publication. Secondly, the model was used to simulate the interaction with benzene, where the oxidative metabolism step (Vmax-ox, see Methods section) was increased by a factor 1.6, as found in benzene-exposed hepatocytes. After induction of CYP2E activity, the TCA levels in blood were significantly increased (Figure VII.7), while TCE levels only marginally decreased. Both Cmax and AUC were predicted to increase by a factor of 1.3 as compared to the model for TCE only. The experimental results and the PBPK model simulations indicate that benzene in the mixtures is likely to increase the TCA formation from TCE.

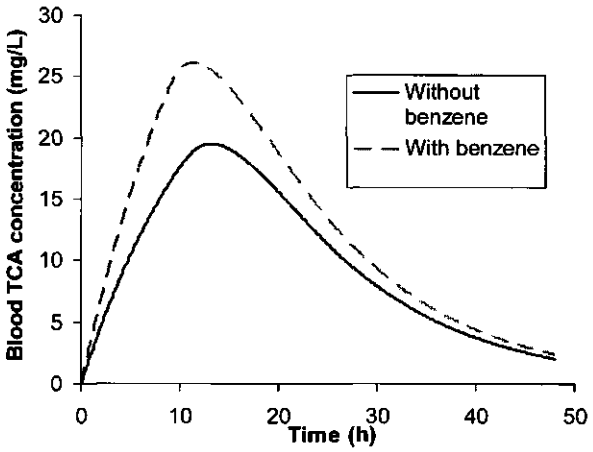

Figure VII.7 *PBPK model simulation for TCE at 2000 mg/kg/day*
Simulated formation of trichloro-acetic acid (TCA) from trichloroethylene (TCE) by hepatic biotransformation. This formation was simulated without, and with (dashed line) the presence of benzene in the exposure. Thus, the dashed line indicates the toxicokinetic interaction between TCE metabolism and benzene on TCE metabolism, which is caused by an increased activity of oxidative metabolism (CYP 2e1 protein activity) due to the presence of benzene in the mixture.

VII. 4 DISCUSSION

The present study demonstrated how investigative toxicological methods enabled to acquire more insights in effects of mixtures of benzene and trichloroethylene. Hypothesised effects of mixtures based on single compound data were tested in the present study.

In vivo subacute toxicity studies

Statistical analysis of the conventional toxicological parameters to assess joint effects and interactions of B and TCE was described earlier by Jonker *et al.* [199]. In summary, the analysis showed no influence of benzene on nephrotoxicity that was dose-dependently induced by TCE. The mixtures increased the relative liver and kidney weight to about the same extent as TCE at the high dose. The benzene-induced hematotoxicity was more severe in the presence of TCE, though only benzene and not TCE was known to induce hematotoxicity [128]. It was proposed earlier [200] that benzene-induced hematotoxicity could be aggravated by TCE because of the ability to induce enzymes for bioactivation of benzene. Thus, the conventional toxicity parameters suggested joint additive effects of B and TCE in most cases, except for the hematotoxicity. However, these parameters could not provide explanations for the joint effects. For this purpose, investigative methods (transcriptomics, *in vitro* hepatocyte exposure and PBPK modelling) were applied that could provide insight in the effects at the molecular level.

Hepatic gene expression profiling (transcriptomics)

Transcriptomics measurements demonstrated effects of mixtures of B and TCE at the gene expression level, and provided insight in mechanisms putatively involved. The effects of TCE largely confirmed previous findings, both by individual gene expression changes, as well as by the entire profile changes, as demonstrated using the PLS models. The coefficient of correlation between the gene expression changes (used to calculate 'predicted exposure levels') and the actual levels of exposure was 0.88. Because the models were built on data of 28-day exposures, the correlations between the predictions and actual dose levels would probably have been higher if the rats in the main study had also been exposed for 28 days. However, the actual duration of the exposure was about 14 days. This could explain why the samples from the main study showed less pronounced gene expression changes than expected. The genes that were affected (table VII.2) were predominantly related to biotransformation, oxidative stress and to beta oxidation of fatty acids, as discussed below. B induced several gene expression changes in common with TCE. For benzene, gene expression changes (individual as well as the profiles analysed with the PLS model) were not very well reproducing the data of the range finding study. In both studies, many gene expression changes were subtle, and a pronounced dose-response was not observed. It is likely that the hematotoxic effects were critical and largely influenced the observations in liver. Other causes of the differences were the unexpectedly severe effects of benzene at the initial concentration of 600 mg/kg/day in the main study, and to differences in the duration of the exposure.

For TCE, a PLS model could be used to predict dose levels based on the expression changes of 400 genes selected in a previous study. Many of the hepatic gene expression changes caused by TCE in the previous study were confirmed in this study. Analysis of gene expression profiles after exposure to mixtures indicated that effects of TCE were enhanced in the presence of benzene. Most of the individual genes that were significantly modulated by

TCE responded to the mixtures in a similar manner, regardless of the presence of benzene. In the cases where both compounds were found to affect the expression, the response to the mixtures could usually be explained roughly by addition of gene expression changes induced by the single compounds. Genes that were induced by both B and TCE indeed were more pronouncedly elevated by the mixtures (eg. Afar). Several genes that were reduced by both compounds showed a more pronounced reduction by the mixtures (e.g. G6pc, Cdh17 and Oat). For a few genes, the response to the mixture (at high doses) deviated from the expectations based on addition of the effects of the individual compounds. The induction of the Cd36 antigen enzyme was markedly higher than expected. This gene, encoding an enzyme related to fatty acid metabolism, was induced by TCE, though not by B. The mixtures of B and TCE at high doses strongly induced Cd36. However, rather than indicating an (synergistic) interaction, this effect could be explained by previous data. In the previous range finding study, B induced Cd36 more than two-fold. [5]. Several genes (incl. ferritin, Gstm and Slc38a2) responded less than expected to the mixtures. Further investigation should demonstrate whether these observations can be ascribed to interactive effects. The effect could for some genes also be ascribed to an upper bound in the increase of gene expression or other transcriptional regulatory mechanisms that affect the gene expression levels.

The observed gene expression changes allowed to generate hypotheses on how B and TCE may elicit joint actions or interactions. Numerous genes differentially regulated in the range finding studies and the present, main study, suggested that biotransformation, fatty acid metabolism and peroxisomal proliferation were most importantly involved in the response to B and TCE (mixtures).

Biotransformation

Toxicokinetic interactions were expected because B and TCE are both metabolised primarily by the CYP2E enzyme. If CYP activities would increase, enhanced bioactivation and aggravated hematotoxic effects of B were considered plausible. *In vivo* gene expression induction for biotransformation enzymes CYP2E and GST were corroborated by exposure of hepatocytes *in vitro*. Exposure to TCE though not benzene increased total GST activity. The 1.5-fold induction of Cyp2e gene expression by benzene found previously [5] was corroborated by increased activity of CYP2E in primary rat hepatocytes. Exact matches for comparison of *in vivo* gene expression and *in vitro* (total) enzyme activity were not expected, because many factors may determine whether transcriptional upregulation (*in vivo*) also leads to increased enzyme activity (*in vitro*). However, present findings were in agreement with each other. The concentrations at which induction occurred *in vitro* (from 0.5 -10 mM) were comparable to liver concentrations *in vivo* as predicted by PBPK models for benzene [210]. The induction of these biotransformation enzymes may be relevant for the toxicokinetics, upon exposure to mixtures of B and TCE.

Although the induction of Cyp2e gene expression by benzene could not be confirmed in the present *in vivo* study, induction of CYP2E enzyme activity was observed after exposing rat hepatocytes *in vitro* to benzene. Moreover, a recent publication reported induction of CYP2E gene and protein expression in rats exposed to benzene [159]. The effect on TCE metabolism of CYP2E induction due to benzene in the mixture was analysed with a PBPK model (see methods section). The metabolism of TCE contains several interacting routes

[204], making it difficult to predict effects of CYP2E induction on the internal concentrations of TCE and its metabolites, e.g. trichloro-acetic acid (TCA). TCE is rapidly exhaled after an oral intake. An increase in CYP2E activity may directly increase the tissue levels of TCEOH and TCA, which are much less volatile. TCA has been shown to induce PPARα and peroxisome proliferation in rodent hepatocytes [211-214] . Because the GST mediated pathway only accounts for minor fraction of the total metabolism, changes in GST activity will probably not alter the disposition of TCE or of its oxidative metabolites. After 1.6-fold induction of CYP2E activity, the TCA levels in blood were found to increase, compared to the model for TCE only. Thus, the results of the PBPK model simulations indicate that benzene in the mixtures is likely to increase the TCA formation from TCE. In conclusion, exposure to mixtures of B and TCE were suggested to enhance levels of TCA, an active metabolite of TCE that most likely determines the toxic effects [213];[215].

Role of the electrophile response element

B and TCE were found to alter expression of many biotransformation enzymes such as Afar, Nqo1, Ephx1, Adhs and Aldhs. The induction of genes like Nqo1, Afar, Gsta, Gclc, ferritin and peroxiredoxin1 is commonly regulated by a mechanism under transcriptional control of the Electrophile Response Element (EpRE or ARE) [177];[[124]; [86-87]. Present data indicate that both B and TCE induced expression of genes through this responsive element, which therefore might be a site for interaction upon simultaneous exposure to both compounds.

Peroxisome proliferation and the role of PPAR in response to TCE

Another observation in the studies with individual components was that B and TCE modulated gene expression related to fatty acid metabolism. TCE pronouncedly modulated enzymes involved in beta oxidation of fatty acids. Most pronounced were the induction of enzymes like dodecenoyl CoA isomerase, Cyp4b, Enoyl-CoA, hydratase/3-hydroxyacyl Co A dehydrogenase and the trifunctional enzyme. In combination with the observed induction of relative liver weight, the gene expression changes provide indications for peroxisome proliferation, that could be mediated by the peroxisome proliferation activated receptor (PPAR alpha). This receptor, in combination with the retinoid X receptor mediates expression of many genes related to, a.o. fatty acid metabolism, including Acetyl CoA oxidase (Aox), Cd36, the bifunctional enzyme and Cyp4. Expression levels of PPAR itself were not determined in our studies. However, the range finding studies and the main study showed that B and TCE (mixtures) induced expression of a.o. Cd36 and Aox. We previously reported that TCE modulated expression of genes in common with reported compounds that induced peroxisome proliferation in rats, such as Wy14,643, Clofibrate and gemfbrozil [6]. Taken together, the effects of TCE on the induction of hepatic peroxisomal proliferation seem to play an important role also when TCE is administered in combination with benzene. The biotransformation of TCE to TCA might be crucial in the determination of hepatic toxicity, as the secondary metabolites of TCE, TCA (and DCA) were found to induce peroxisome proliferation in rodents (though not in humans) [212];[214]. The effects of benzene on bioactivation of TCE to TCA (with implications for peroxisomal proliferation) were further analysed using an *"in silico"* PBPK model, as described below. Further, dedicated studies could be designed to effectively demonstrate the role of PPAR alpha and induction of peroxisomal proliferation and putative interative effects upon exposure to mixtures of B and TCE.

Other cellular mechanisms

Besides biotransformation and peroxisomal proliferation, B and TCE (mixtures) seemed to affect various cellular processes. These findings could not always be fully understood and incorporated in a coherent and conclusive description of the mechanism of combined action of B and TCE. Nevertheless, they may provide useful starting hypotheses for further investigations. Therefore, a selection of the findings is briefly presented.

Both compounds in the range finding studies induced hepatic GSH levels, and gene expression of Gclc and Gclm, subunits of the key enzyme for biosynthesis of GSH. In response to mixtures, Gclc induction was not confirmed with statistical significance. However, GSH synthase, another important enzyme in GSH biosynthesis, was induced by the mixtures.

Data both from clinical chemistry and transcriptomics analyses suggested changes in cholesterol metabolism upon exposure to B and/or TCE. In the range finding studies, B induced gene expression of HMG-CoA reductase, a key enzyme in cholesterol biosynthesis. B also slightly induced plasma cholesterol levels (n.s.) in the range finding studies, but the main study showed slightly reduced levels. At high doses, B and TCE induced expression of 7-dehydrocholesterol (Dhcr7) reductase, which is involved in regulation of cholesterol biosynthesis [216-217].

Protein synthesis and degradation were affected at the transcriptional level by both compounds. TCE was found to markedly change expression of genes related to the hepatic acute phase response. Possibly associated to changes in glycolysis and/or gluconeogenesis, B modulated expression of the gene transcription regulator Hnf4 (and Hnf6), and both B and TCE reduced expression of G6pc. Another hepatocyte nuclear factor (Hnf3 gamma) was reduced in the liver samples of rats exposed to TCE and the mixtures with high TCE concentrations. Hnf3 has been associated with a.o. regulation of expression of tyrosine aminotransferase (Tat) [218], in the glucocorticoid response [219]. Tat was reduced by both TCE and benzene and slightly (n.s.) by the mixtures. Previosuly, elevation of Hnf3 gamma levels was found to attenuate the acute liver injury caused by administration of CCl4 [220]. Furthermore, Hnf 3 regulates human Cyp2c [221]. The rat Cyp2c12 expression was also associated to this transcription factor [222]. In agreement, our study showed reduced expression of Cyp2c12 by B, TCE and mixtures. The putative involvement of more processes was shown by expression changes of e.g. Cdh17 (cell adhesion), thyroid hormone responsive proteins, kidney-specific protein and alpha albumin.

Conclusions

This subacute toxicity study analysed effects of mixtures of B and TCE in rats in a stepwise approach. Hypotheses were generated based on effects of the single compounds, and tested in rats exposed to mixtures. Profile analysis with multivariate statistics exploited the large transcriptomics datasets to provide indications for enhanced hepatic TCE-effects in the presence of benzene. Transcriptomics data confirmed previously reported induction of genes related to biotransformation, but also highlighted changes in genes related to various other processes such as TCE-induced peroxisome proliferation. Putative roles of transcriptional regulation mechanisms involving EpRE, PPAR, Hnf and others were highlighted in the response to B and TCE and dedicated studies for this can now be designed.

When hepatocytes were exposed *in vitro* of to benzene, CYP2E enzyme activity increased. According to *in silico* PBPK modelling, this induction enhanced oxidative bioactivation of TCE to TCA, which presumably is responsible for the toxic action. Thus, induction of CYP2E by benzene is one plausible explanation for the toxic effects of mixtures of B and TCE. Further studies could refine the PBPK models and the effects of changes in other biotransformation enzymes. For instance, more research could clarify the role of GSH conjugation by GST isozymes with subsequent toxicity and/or carcinogenicity in the kidneys. In addition, PBPK models for benzene could aid to identify the implications of the presence of trichloroethylene.

In summary, this study demonstrates that an integrated approach with investigative toxicological methods including transcriptomics enabled better understanding of joint effects of chemicals upon exposure to mixtures.

Acknowledgements

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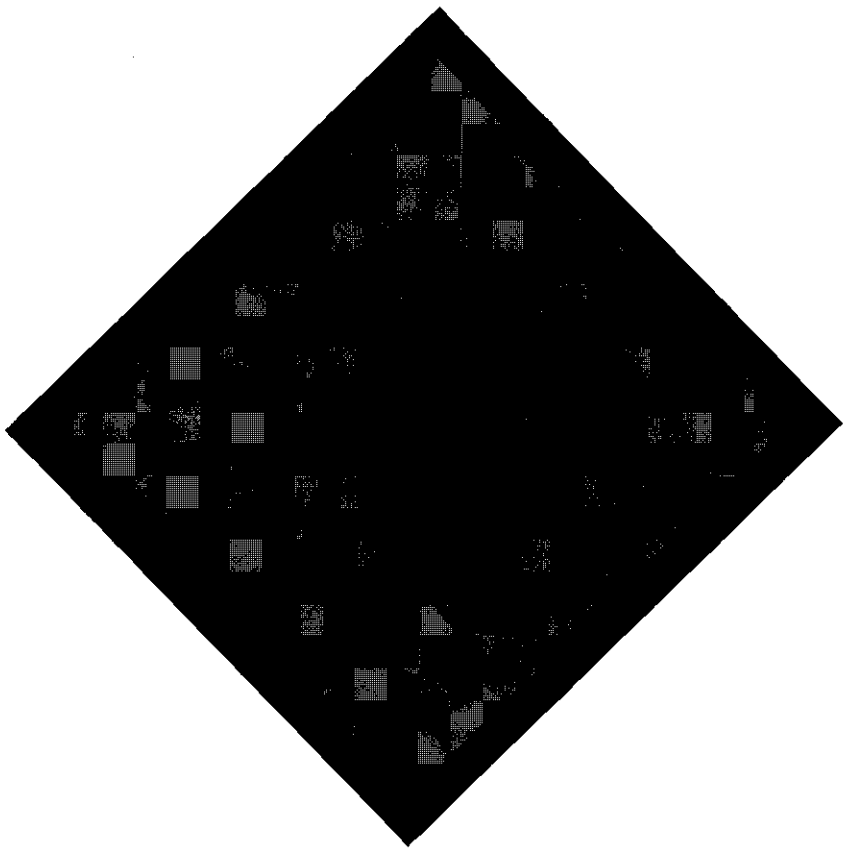
Chapter VIII

Discussion and conclusions



Chapter VIII

VIII. Discussion and conclusions

Contents

VIII. Discussion and conclusions

The studies described in this thesis were performed to explore applications of toxicogenomics. The studies primarily analysed the effects of chemicals in rat liver. The results of the studies will be briefly summarised. Potential applications of toxicogenomics will be discussed using results of the studies described in chapters II – VII. The technical aspects like sensitivity and reproducibility of the toxicogenomics methods are evaluated, as well as the relation between the different technologies. Challenges like the "linking of the layers" of gene, protein and metabolite measurements are mentioned. Lastly, the perspectives of toxicogenomics for hazard characterisation and human health risk assessment are discussed.

VIII.1 Summary of the results

Toxicogenomics methods were used to study the response in rat liver after exposure to bromobenzene, benzene and trichloroethylene and mixtures. Chapter II described transcriptomics and proteomics analyses in livers of rats exposed to bromobenzene. The protein and gene expression changes, 24 hours after single dosage of this well-known model hepatotoxicant, supported existing and raised new hypotheses about mechanisms of hepatotoxicity. The crucial role of hepatic glutathione levels and oxidative stress was apparent, and a cluster of genes was found to be co-ordinately induced by the electrophile response element (EpRE, also ARE). Gsta induction was confirmed by increased activity of this enzyme in cytosolic fractions. Induction of aldehyde dehydrogenases at both the gene and protein level might be related to lipid peroxidation by bromobenzene. Changes were related to metabolism of fatty acids and cholesterol, protein synthesis and proteolysis. Bromobenzene was found to elicit an acute phase response. The proteomics results corroborated findings like increased proteolysis. Proteomics and transcriptomics provided complementary results, and further development of proteomics methods could enhance analysis of correlations between gene expression and protein changes.
Chapter III demonstrated the dose and time dependency of the gene expression changes by bromobenzene. Liver samples were measured at 6, 24 and 48 hours after dosage. With transcriptomics, effects were detected earlier and at lower dose levels compared to histopathology and clinical chemistry. Several gene expression changes were confirmed with the branched DNA assay. Markers for oxidative stress like heme oxygenase were induced already 6 hours after dosage. The suggestion that glutathione has a key role in the response to reactive metabolites of bromobenzene was supported by the finding of GSH synthesis and induction of enzymes responsible for this.
In chapter IV, the transcriptomics data were analysed in combination with metabolomics analysis of urine and blood plasma of the same rats exposed to bromobenzene. The combined analysis of hepatic transcriptome and plasma metabolome data provided more insights in chemically induced hepatotoxicity, eg. in the involvement of apoptosis. The degree of hepatic necrosis in individual animals could be correlated to single metabolite signals and gene expression changes, using multivariate statistical techniques.
Chapters II, III and IV described the effects of a model toxicant that induced obvious signs of liver damage within days. The question remained whether gene expression changes were characteristic for hepatotoxicity. Therefore, the next studies analysed hepatic gene expression changes in response to compounds which were not model hepatotoxicants. In chapter V and VI, effects at the liver gene expression level were reported after exposure

for 28 days to different concentrations of the widely used chemicals benzene and trichloroethylene (TCE). Transcriptomics analyses showed many hepatic gene expression changes, though histopathology and clinical chemistry did not reveal abnormalities in liver. Gene expression changes related to hepatic biotransformation of both TCE and benzene were found, which may influence the toxicity of benzene, in blood and bone marrow, and of TCE in the kidneys.

Benzene-induced changes were compared to effects of the model hepatotoxicants bromobenzene and APAP, which induced hepatic necrosis. While results partly overlapped, the effects of benzene were more subtle, both in the magnitude and the number of the changes. In the next study, TCE was found to provoke changes similar to benzene, though also for TCE, a characteristic profile of gene expression was obtained. For instance, characteristic gene expression changes corroborated the peroxisome proliferating capacity of TCE. In conclusion, the studies enabled to delineate characteristic gene expression changes upon exposure to (potentially) hepatotoxic chemicals. Metabolomics analysis or urine (NMR and multivariate statistics) enabled to assess exposure to benzene and TCE, even low dose exposure.

In chapter VII, toxic effects of mixtures of benzene and trichloroethylene in liver were analysed. To our knowledge, this was the first study to investigate combined action of mixtures of (toxic) compounds at the gene expression level. Livers of rats, exposed to benzene, trichloroethylene or to mixtures of these chemicals at different dose-levels were analysed with transcriptomics. Both individual gene expression changes and changes in the profiles of gene expression, analysed with multivariate statistical models, showed that benzene enhanced the effects of TCE.

VIII.2 Applications of toxicogenomics

From the applications of toxicogenomics that were discussed in the introduction, this thesis primarily demonstrated the use of toxicogenomics to:

d) **investigate mechanisms of toxicity** (see I.2.1)

e) **obtain new markers of toxicity** (see I.2.2)

f) **assess effects of mixtures of toxic compounds** (see I.2.4)

In the following section, each of these applications is discussed in more detail in relation to the studies described in this thesis.

a) mechanisms of toxicity

Initial studies were performed to characterise mechanisms of toxicity at the protein and gene expression level in liver, in relation to pathological endpoints. Hepatic necrosis was induced by the well-studied compound bromobenzene, and many genes in the liver were found to be differentially expressed. The changes were related to biological processes like biotransformation, GSH metabolism, oxidative stress, acute phase response. Bromobenzene pronouncedly induced epoxide hydrolase (Ephx1) and Glutathione-S-transferase alpha (Gsta), which could enhance detoxification of harmful metabolites of bromobenzene. The response to highly electrophilic metabolites of BB was suggestively mediated by the electrophile or antioxidant response element (EpRE or ARE). Also unexpected processes were affected, like cholesterol and fatty acid metabolism, protein synthesis and proteolysis. The combination of hepatic transcriptomics and metabolomics analysis of plasma (chapter IV) revealed more details in the mechanism of hepatotoxicity, and showed relationships between changes in expression of hepatic enzymes and metabolite levels in blood. In contrast to plasma, the profiling of urine did not contribute very much to the elucidation of mechanisms of biotransformation and toxicity of bromobenzene, nor of benzene or TCE, primarily because not enough signals could be identified.

In the studies with benzene and TCE, transcriptomics enabled to identify effects relevant for the mechanism of toxicity, such as induction of bioactivating enzymes. Benzene and TCE were also found to affect the metabolism of fatty acids and sterols, including cholesterol. An interesting finding was that TCE reduced hepatic expression of alpha 2u-globulin, which possibly explained why hyalin droplet nephropathy was not found with TCE, and could be refuted as a possible mechanism for nephrocarcinogenicity of TCE in rats. The known capacity of TCE to induce peroxisome proliferation was corroborated by relative liver weight increase and induction of gene characteristic for this effect. These genes were also found in studies with peroxisome proliferating agents.

The toxicogenomics measurements revealed a multitude of effects. Adaptive responses, reversible effects and "normal" physiological effects like intraday variation should be distinguished from specific effects of the toxic stimuli. To delineate the significance and specificity of the effects, the gene expression changes induced by bromobenzene were compared with reported effects of high doses of acetaminophen (APAP, paracetamol) well-known to be hepatotoxic. An overlap in gene expression changes was observed,

especially in the pathways that were altered. Subsequently, gene expression changes were compared to effects of benzene and trichloroethylene. These widely used chemicals are not well-known hepatotoxicants. However, both compounds induced relative liver weights and GSH levels. Other effects in liver were not observed with histopathology and clinical chemistry. However, expression of many hepatic genes was modulated both by benzene and TCE. Several genes were modulated commonly by B and TCE, and a few effects were seen with benzene, TCE and with the model hepatotoxicants. Nevertheless, all compounds tested revealed many specific gene expression profiles changes, indicating that transcriptomics is useful to investigate and discriminate the effects of different (potentially) hepatotoxic compounds that lead to different (adverse) effects in the liver. In conclusion, the studies showed that toxicogenomics, especially transcriptomics, delineated crucial steps in the mechanisms of toxicity. Gene expression changes could be related to known effects of the compounds, while also many yet unknown effects were discovered.

The transcriptomics studies in this thesis, as well as in other laboratories, showed that a fraction of the more than 30000 (rat) genes was differentially expressed in liver by many different treatments. As might have been expected, genes highly expressed in liver (e.g. biotransformation enzymes) were identified in many of the toxicogenomics studies. To this moment, studies predominantly analysed livers, and a large part of the important effects in the liver could be determined by measurement of a limited number (hundreds) of genes. The analysis of other tissues would probably require measurement of other genes. Identification of putatively interesting and/or unexpected effects would require measurements of as many genes as possible.

b) ***new markers of toxicity***

Transcriptomics, proteomics and metabolomics were applied to obtain new markers of hepatotoxicity, for early identification of effects, at exposure to lower dose levels in comparison to the current markers. The methods identified gene, protein and metabolite changes in relation to the bromobenzene treatment (Chapter II - IV). Some effects at the gene expression level were identified early (6 hours) after treatment, when no other toxicity markers demonstrated effects. Many of the BB-induced effects were seen at a dose level 2,5 to 10 - fold below the level that induced effects observed with the conventional methods. The combined analysis of hepatic transcriptome and plasma metabolome yielded a ranked list of potential gene expression and metabolite markers of hepatic necrosis (Chapter IV). The potential, early and more sensitive markers of hepatotoxicity, should be further validated for use in assessment and quantification of hepatic necrosis. Metabolomics profiling revealed characteristic patterns of metabolites in urine. Because the identity of the individual signals was not established, individual metabolites as markers of benzene or TCE toxicity were not obtained. However, the entire profiles could be used to specifically and robustly assess exposure and/or effects of benzene and TCE (Chapter V and VI). In the study with mixtures of benzene and TCE (chapter VII), gene expression changes were found that could serve as markers to facilitate assessment of interaction effects upon exposure to mixtures. In conclusion, toxicogenomics methods yielded potential markers that discriminated treated from control samples, and markers that correlated with the degree of necrosis. Nevertheless, studies have to be performed to validate and establish these markers for use in routine practice.

c) ***effects of mixtures of toxic compounds***

The study described in chapter VII investigated effects of mixtures of benzene and TCE, at the gene expression level. Subacute toxicity and gene expression changes were analysed in samples exposed to benzene, TCE or mixtures. Effects of the previous range finding studies (chapter V and VI) were largely confirmed. The study demonstrated that various gene expression changes induced in liver by TCE were enhanced by benzene. Most of the genes responded as expected by combining the effects of the individual compounds. The measurements provided insights in biological pathways and regulatory mechanisms in the cell that were involved in the response to the exposures, such as the electrophile response element (EpRE,ARE), the peroxisome proliferation activated receptor (PPAR alpha) and a hepatic nuclear factor (Hnf3). The profiles of hepatic gene expression, used in multivariate statistical PLS models, proved also useful to assess the effects of the mixtures. The mathematical models also provided indications that benzene enhanced the TCE-induced changes at the hepatic gene expression level.

A transcriptomics study was initiated in our laboratory to identify effects of mixtures of mildly hepatotoxic food additives [223], [224]. These additives were curcumin, propyl gallate, butylated hydroxytoluene and thiabendazole. These compounds induced relative liver weights, but no overt hepatotoxic effect. The first studies with 28-day exposure to the individual components provided new insights in the mechanisms of action at the gene expression level. The effects of combined intake of these food additives will be tested in further studies.

The studies to assess mixture effects so far indicated that effects upon exposure to mixtures of toxicants might be subtly different from single compounds, and that experimental designs have to be chosen carefully to demonstrate interactions. Preferably, single compound, as well as mixture exposures at several dose levels should be included and performed in one integrated experiment.

Taken together, transcriptomics may enhance the assessment of mixture toxicity, by analysis of individual gene expression changes, as well as by multivariate statistical analysis of profile changes.

Other applications of toxicogenomics

As explained in the introduction, toxicogenomics results could facilitate interspecies and intraspecies extrapolation. Studies in (especially chapter VII) have demonstrated interindividual variation in gene expression in response to toxic chemicals. Thus, toxicogenomics methods might enable to better characterise why individuals respond differently to treatments. Toxicogenetics studies the genetic basis for interindividual differences in response [225], and may be used together with transcriptomics, to studies the relation of genetic differences to expression of the genes. Only limited amounts of data are available, and more research is required to determine which genes show the largest interindividual variations (in composition and expression), and what the implications of these variations are for development of toxic effects. Toxicogenomics may build upon the extensive research that has already been performed to identify interindividual and interspecies differences in (mainly) drug metabolising enzymes and the consequences for the metabolism of xenobiotics. It is important to note that (routine) toxicity studies are performed with inbred strains of rats and mice, largely reducing the

differences in genetic background. The differences in response in humans might therefore be much larger than in animal experiments.
The studies provided some indications that toxicogenomics could reduce, refine or replace the use of laboratory animals. It might support alternatives to animal testing, predominantly by providing detailed insights in mechanisms of toxic action in *in vitro* experiments. The insights may be used to refine *in vivo* toxicity testing. Because toxicogenomics can investigate many parameters in studies *in vitro*, this may increase the value of models to assess, explain and even predict toxicity *in vivo.*

VIII. 3 Technical aspects of toxicogenomics methods

Various technical aspects of the toxicogenomics methods (especially transcriptomics) were evaluated. The relation between transcriptomics, proteomics and metabolomics, and the strengths and limitations of each of these technologies was assessed. The sensitivity was evaluated in relation to conventional methods in toxicology, as described in the next section. The reproducibility of toxicogenomics studies was determined and efforts to standardise toxicogenomics measurements worldwide are discussed.

Relation between functional genomics technologies
The studies in this thesis applied transcriptomics, but also proteomics, and metabolomics in toxicolgical experiments. Measuring gene expression by mRNA levels using DNA microarrays has proven to provide useful information for toxicology. However, mRNA molecules have the indirect function to transfer information, rather than to directly act in cellular processes. In the first study described in this thesis (chapter II) transcriptomics results were directly related to proteomics measurements of the same livers. Thus, the correlation between gene expression and protein levels was investigated. Technical limitations of the proteomics technolgies hampered the finding of many changes in protein levels to corroborate observed changes in gene expression. Especially the reproducibility of gelelectrophoresis and the identification of proteins with mass spectrometry limited the success of this approach. Nevertheless, complementary information was obtained from both techniques, for instance regarding the involvement of processes like increased protein synthesis and breakdown. With further developments and optimisations of proteomics technologies, these will prove to be of more importance for the elucidation of molecular mechanisms, because many cellular processes are influenced at the protein level. Subcellular redistribution, protein interactions and post-translational modifications such as phosphorylation may be crucial for correct functioning of cellular processes.
Profiles of metabolite contents of tissue samples or body fluids such as blood plasma or urine could identify changes in biochemical processes in the samples, which may relate to effects in specific tissues or entire organisms. In contrast to transcriptomics and proteomics, metabolomics can provide evidence, rather than indications, of changes in biochemical pathways. Metabolomics was applied in studies described in chapter IV, V and VI. At present, the major shortcomings of metabolomics are the limitations to the identification of individual constituents of the metabolite profiles, and to the (mass) range of molecules that can be detected. A major strength of metabolomics, and of proteomics, is that samples (urine, saliva) can be used that are collected by non-invasive methods, while these samples are not useful for mRNA measurements with transcriptomics.

Sensitivity of toxicogenomics methods

The sensitivity of toxicogenomics methods for the detection of effects was compared to that of conventional toxicity markers in several of the studies. Transcriptomics using DNA microarrays proved to be more sensitive than the conventional toxicity markers to detect effects. It is important to note that for most of the (gene expression) changes, it remains to be clarified whether these are irreversible and adverse effects. Some expression changes were identified early (6 hours) after treatment, when conventional toxicology could not demonstrate effects. Many of the BB-induced effects were seen at dose levels2,5 to 10 - fold below the levels that induced effects observed with the conventional methods. Also after exposure to benzene or trichloroethylene, DNA microarrays were able to show many significant changes in gene expression at the lowest dose levels tested, while conventional toxicity markers detected effects on at 10 to 20-fold higher dose levels.

Interindividual changes in gene expression within treatment groups were observed. After exposure to BB, animals exhibited different degrees of hepatocellular necrosis, which was also recognised at the gene expression level. Besides this, interindividual changes in gene expression were observed in the BB studies as well as in the mixtures study with benzene and TCE (in chapter VII), in the absence of overt physiological differences between the rats.

Also, effects of corn oil (vehicle control)could be recognised with transcriptomics methods, as shown in table II.8. Furthermore, DNA microarrays enabled to demonstrate differences in liver mRNA levels of untreated rats, depending on the time of sacrifice. Liver samples isolated 24 or 48 hours after start of the study were more similar to each other than to samples isolated 6 hours after start of the study. Thus, this method may enable to characterise intra-day changes in healthy animals in detail.

The sensitivity of proteomics methods for identification of single proteins was lower than current methods like immunohistochemistry (ELISA or Western blotting). However, in contrast to those methods, proteomics enable to detect changes in unexpected and unknown proteins. For identification with mass spectrometry, a minimal amount of protein has to be available (recovered from the gels), which is not always possible. With the 2D-gel based proteomics method, typically only water-soluble proteins within a specified mass and pI range will be separated. Membrane-bound proteins will therefore not be identified. Furthermore, proteins that exactly co-migrate may mask each other on the gel. The analysis of whole proteome patterns with powerful image analysis software and multivariate statistical methods enhances the sensitivity, and the specificity of (2D-gel based) proteomics measurements.

The metabolomics applications described in this thesis showed that methods with NMR analysis of urine and plasma, in combination with multivariate statistical methods proved more sensitive than the current toxicity markers. Using multivariate statistical tools for pattern recognition of the NMR spectra, nearly all samples exposed to the lowest dose levels tested in the different studies could be distinguished from control samples.

Reproducibility of toxicogenomics methods

The reproducibility of transcriptomics methods is illustrated by the studies described in this thesis. Within studies, stringent quality criteria proved indispensable to obtain reproducible

data. After application of these criteria, replicate DNA microarrays of identical samples and samples of the same treatment groups generated comparable results, with variation smaller than the treatment effects.
Inter-study reproducibility was shown from results in chapters II and III. Both studies with exposure of rats to bromobenzene were performed independently. There were several differences in the protocols, such as the route of administration. Moreover, different batches of in-house produced cDNA microarrays, with partially different contents, were used for the two studies. Nonetheless, the effects measured 24 hours after dosage were largely overlapping. Especially the studies with trichloroethylene, reported in chapter VI and VII, showed reproducible gene expression changes. The results from the range finding studies with TCE in chapter VI proved suitable for the construction of a mathematical model that could describe and predict effects of TCE in the subsequent main study, described in chapter VII. Another confirmation comes from the comparison (chapter VI) between effects of TCE and agents that induce peroxisome proliferation, reported by several other laboratories.
The studies with B and TCE made use of oligonucleotide DNA microarrays produced in collaboration with the Genomics lab at the Utrecht University Medical Centre (Utrecht, NL). Unpublished results with samples of the bromobenzene study showed good correlation of the results obtained with these oligonucleotide DNA microarrays when compared to the previously (chapter II, III) used cDNA microarrays.
Inter-laboratory reproducibility was shown by comparison of results from our laboratory with results of the NIEHS laboratory (U.S.A. National Institute of Environmental Health Sciences) laboratory [69]. Expression of various genes changed in the same manner in rat livers after bromobenzene exposure in our laboratory, as compared to exposure to high doses of acetaminophen, at the NIEHS. The similarities were especially clear when the biological processes that were affected were compared.
The International Life Science Institute consortium (ILSI) analysed the inter-laboratory variation in transcriptomics experiments, and reported that "despite some variability, robust gene expression changes were consistent between laboratory" [226].
Presently, large, worldwide efforts are undertaken to standardise the DNA microarray production technologies, the experimental protocols and data analysis to enhance inter-study comparisons. MIAME was initiated to define the minimal information that should be provided with microarray experiments to enable interpretation and sharing of data between researchers (MIAME, www.mged.org). Databases are created to uniformly accommodate gene expression profiles gathered throughout the world, for instance at the European Bioinformatics Institute (TOX-Express) and at the US National Centre for Biotechnology Information (NCBI, GEO). The U.S. centre for toxicogenomics (NCT) established a database named (CEBS) to specifically accommodate toxicogenomics data. The Netherlands Toxicogenomics Centre (NTC) was founded to facilitate collaborative toxicogenomics efforts within the Netherlands (TNO, RIVM, Rikilt, University of Maastricht). It has become clear that global standardisation and collection of data into relational databases is required to enable further developments of toxicogenomics towards successful applications in toxicological hazard and risk assessment.

In this thesis, the reproducibility of proteomics methods was only evaluated for the 2D-gel based method in the BB study described in chapter II. This demonstrated considerable technical variation between the three replicate gels per sample. Therefore, statistical

analysis could only confirm changes in protein levels for 24 proteins, while, even at a glance, many more protein spots seemed to be affected by BB treatment. The labour-intensive methods using 2D-gels required improvements, because most applications required not only qualitative but also quantitative assessment of proteins in a sample. Studies with updated or alternative methods (eg. MS-MS) for proteomics research showed better reproducibility and more promising results [227-232].

The assessment of the reproducibility or metabolomics measurements in this thesis was restricted to technical and interindividual replications of the NMR spectra. By default, urine of plasma samples were recorded in triplicate with NMR spectroscopy, for all animals in the studies. In general, the technical variation between the spectra was smaller than the experimental, or interindividual variation. Comparisons of spectra between groups treated with different dose levels showed that the same signals recurred, though in different quantities, dependent on the dose levels. In conclusion, triplicate technical replicates for every individual sample in the study are sufficient to demonstrate experimental effects with NMR-based metabolomics methods.

In conclusion, the toxicogenomics methods each provide a wealth of (detailed) information, and are largely complementary to each other. Currently, especially transcriptomics and metabolomics are sensitive and reproducible, while proteomics methods may become so after further developments. Initiatives are taken to standardise the methods to enable information exchange and comparisons of toxicogenomics studies. At this moment, transcriptomics is probably the most powerful and easy to apply of the functional genomics technologies. Proteomics and metabolomics will prove to be more relevant in the (near) future, given that some technical limitations are resolved.

VIII.4 Challenges and perspectives in toxicogenomics

Confirmation of hypotheses

The functional genomics technologies produce vast amounts of data, typically with a somewhat lower level of confidence compared molecular biological measurements of single genes or proteins. Therefore, it is recommended that these technologies are used to test, but to generate hypotheses. These hypotheses should be confirmed and further investigated with dedicated methods by researchers with specific expertise and equipment. Changes in gene and protein expression or metabolite contents found with toxicogenomics methods can be designated as adverse effects only after correlation to physiological effects that confirm toxicity.

Linking the layers

The challenge for the coming years is to study interactions between genes, proteins and metabolites and to integrate the results of the different functional genomics methods ("linking the layers"). Linking the measurements of genes, gene expressions, proteins and metabolites could provide the insights required to understand the processes within a cell. It is essential to apply multivariate statistical techniques to resolve the connections between the different data, and to be able to introduce the aspect of time to recognise and

explain dynamic effects in the processes. In toxicology, the influence of duration of exposure, time of recovery before analysis and the exposure dose levels are crucial determinants of the endpoints that may be observed. Better understanding of the sequence of events at the molecular level may enable to extrapolate from (limited) experimental conditions, to assess the toxicity of a compound after realistic exposure conditions (chronic exposure to low dose levels).

Toxicogenomics for human health risk assessment

Toxicogenomics methods may enhance human health risk assessment (see figure I.1) in various ways. Detailed insights in mechanisms of action could be obtained, revealing the crucial steps and sequence of events at the molecular level. The methods enabled more sensitive and earlier detection of adverse effects in (animal) toxicity studies. Furthermore, the identification of effects after exposure to mixtures was enhanced. The value of toxicogenomics for risk assessment was recently analysed by our institute's department of toxicological risk assessment [233]. Also a U.S. society of toxicology taskforce evaluated the use of genomic data in risk assessment [234]. Toxicogenomics may be used for risk assessment given that the significance of the observed effects can be designated. The characteristics of changes in gene expression, protein or metabolite patterns have to be determined and related to conventional end points in toxicity. The effects of many different (model) compounds and treatment conditions have to be compared. For the identification of (no observed) adverse effect levels, it is essential to be able to differentiate adverse effects from reversible, adaptive responses and effects independent of the treatment (eg. intraday variation). Standardisation of the methods and the development of global databases is required to be able to compare toxicogenomics data, especially when patterns are compared to each other. In the absence of these databases, toxicogenomics methods are useful for the detailed investigation of mechanisms of action of particular compounds in dedicated studies. In this respect, toxicogenomics can be advantageously applied to simultaneously evaluate efficacy (e.g. of candidate drugs) and safety of a product (risk-benefit assessment).

Many aspects will eventually determine whether toxicogenomics will be successfully applied in toxicological risk assessment. Regulations and guidelines, standardisation, a public opinion against animal testing and, of course, cost aspects. It was demonstrated in several applications described in this thesis that toxicogenomics can enhance identification and characterisation of toxicity.

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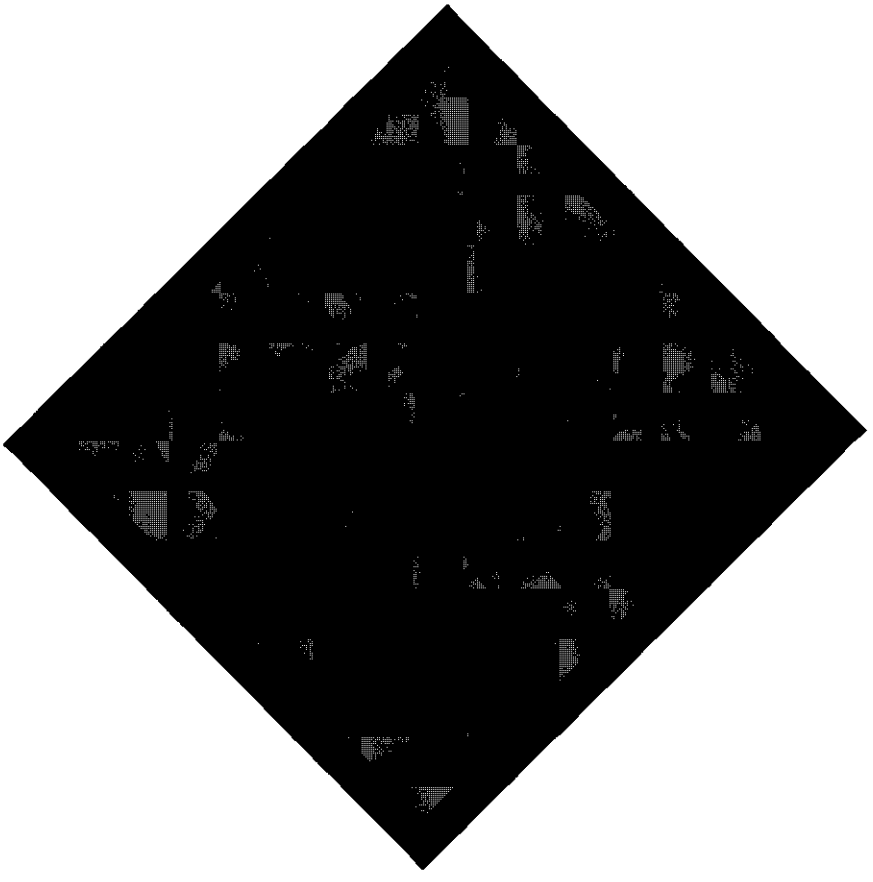

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Shimano	2002	[118]
Slitt	2003	[105]
Smilde	2003	[127]
Smith	2000	[174]
Snyder	1994	[136]
Sowden	2002	[170]
Spellman	1998	[45]
Starek	1991	[209]
Steiner	2000	[17]
Stierum	2004	[223]
Stults	1995	[56]
Su	1997	[205]
Suffredini	1999	[93]
Sugawara	2000	[206]
Sun	1990	[140]
Szymanska	1991	[112]
Szymanska	1998	[95]
Thimmulappa	2002	[123]
Thor	1981	[74]
Tsuji	2000	[87]
Ulrich	2003	[164]
van de Peppel	2003	[149]
Van Duuren	1989	[184]
Vanden Heuvel	1999	[189]
Verhoeckx	2004	[232]
Walgren	2000	[214]
Walgren	2004	[213]

First Author	Date	Ref
Wan	2002	[161]
Wang	1998	[99]
Wang	2002	[113]
Waring	2001	[22]
Waring	2001	[40]
Waring	2004	[226]
Warnock	2004	[228]
Waters	2002	[33]
Waters	2003	[234]
WHO	2000	[179]
Witz	1990	[143]
Wong	1981	[111]
Wong	2002	[193]
Xiong	2002	[109]
Yamamoto	2002	[194]
Yang	2002	[103]
Yen	1999	[172]
Yoon	2002	[163]
Yoon	2003	[175]
Yoshida	2001	[71]
Zhang	1998	[173]
Zhu	2003	[229]

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Appendix of chapter III

Tables also available online at *www.toxsci.org*

Table III. 2. *Gene expression changes 24 h after bromobenzene dosage*

The subset of genes that were significantly ($p < 0.01$) differentially expressed after 24 h in the high BB dosed rats compared to the controls. Genes were categorised according to biological mechanism or pathways. The log (base 2) average fold change and the p-value for the three dose levels are listed. A treshold was chosen for display reasons which was a log (base2) fold change > 0.7 or < -0.7 in any of the dose groups compared to the controls. Statistical level of significance: **: $p < 0.01$, ***: $p < 0.001$. ACC#: Genbank Accession number, metab: metabolism

Category	Gene name	ACC #	Low		Mid		High	
Acute phase	Orosomucoid 1	AI029162	-0.09		0.57		2.05	**
Acute phase; ox.stress	ferritin, heavy polypeptide 1	AA817701	0.33		1.01	**	1.71	***
Acute phase; ox.stress	ferritin, heavy polypeptide 1	AA818441	0.25		0.97		1.53	***
Acute phase; ox.stress	ferritin light chain 1	AA817693	0.48		1.13	**	1.21	**
Acute phase	vitronectin	AA956238	-0.02		-0.41		-0.73	**
Acute phase?	sim to complement subcomponent C1s	AA900592	-0.27		-0.13		-1.01	***
Acute phase	complement component 4a	AA965125	-0.15		-0.59		-1.19	**
Acute phase?	Fibrinogen, B beta polypeptide	AI071033	0.20		-0.11		-1.20	***
Acute phase	alpha(1)-inhibitor 3, variant I	AA817963	-0.47		-0.63	**	-1.21	***
Acute phase	alpha(1)-inhibitor 3, variant I	AA817963	-0.54	**	-0.79	**	-1.42	***
ox.stress	selenoprotein P, plasma, 1	AA963445	-0.11		-0.26		-1.67	***
Acute phase	Serine protease inhibitor	AA901050	-0.01		-0.36		-1.75	**
Ox.stress	Heme oxygenase (HO-1)	AA874884	-0.02		0.14		2.08	***
Ox.stress	Heme oxygenase (HO-1)	AA874884	-0.09		0.12		1.81	**
Ox.stress	tissue inhibitor of metalloproteinase 1	AA957593	-0.17		-0.01		1.49	**
Ox.stress	peroxiredoxin 1	AA875245	0.03		0.92	**	1.29	***
Ox.stress	peroxiredoxin 1	AA875245	-0.05		0.97		1.13	**
Amino acid	argininosuccinate lyase	AA818673	0.06		-0.27		-1.21	**
Apoptosis	cytochrome c, somatic	AA866442	-0.11		0.50		1.00	***
Cholesterol	3-HMG-Co A synthase 2	AI136048	0.25		-0.17		-1.80	**
Cysteine	S-adenosylhomocysteine hydrolase	AA955402	-0.19		-0.35		-0.90	**
Cysteine	cytosolic cysteine dioxygenase 1	AA818579	0.03		-0.29	**	-1.07	**
Drug	epoxide hydrolase 1 (mEH)	AA900551	0.44		2.45	**	2.58	***
Drug;GSH	glutathione S-transferase, alpha 1 (Gsta)	AA818339	0.82		2.47	**	2.27	***
Drug	aflatoxin B1 aldehyde reductase (Afar)	AA923966	0.31		2.15	**	1.94	***
Drug;GSH	glutathione S-transferase, alpha 1 (Gsta)	AA818339	0.62		2.08	**	1.67	***
Drug;GSH	GSTM, soluble,class Mu; (Gstm2)	AA998734	0.48		1.86	**	1.41	***
Drug	sulfotransferase, hydroxysteroid prefer. 2	AA818024	-0.25		-0.57	**	-0.76	**
Drug	Mod. sim to NAD(P)H dehydrogenase (quinone)2	AI029553	-0.11		0.22		-0.83	**
Drug	Cytochrome P450 15-beta Cyp2C12	AA818124	-0.20		-0.25		-0.88	**
Drug	sulfotransferase family 1A, phenol-preferring, member 1	AA866493	-0.17		-0.28		-0.90	
Drug	cytochrome P450, 2c39 (Cyp2C39)	AA818043	-0.15		-0.28		-0.96	**
Drug	arachidonic acid epoxygenase	AA819756	-0.24		-0.29		-1.30	***

Drug	cytochrome P450, 2E1 (Cyp2E1)	AA818896	-0.24	-0.23	-1.36	***
Drug	cytochrome P450 4A3 (Cyp4A3)	AA924591	-0.11	-0.31	-1.37	***
Drug	cytochrome P450, 4a10 (Cyp4A10)	AA956787	-0.01	-0.57	-1.63	**
Fatty acid	Hadha; trifunctional protein	AA900458	0.00	-0.28	-0.74	**
Fatty acid	2,4-dienoyl CoA reductase 1, mit.	AA875267	0.11	0.22	-0.85	**
Fatty acid	delta3, delta2-enoyl-CoA isomerase	AA997009	-0.16	-0.49	-0.86	**
Fatty acid	acetyl-coenzyme A dehydrogenase, medium chain	AA925220	-0.09	-0.55	-0.89	**
Fatty acid	peroxisomal multifunctional enzyme II	AA874974	-0.07	0.18	-0.95	**
Fatty acid	acyl-coA oxidase	AA924697	-0.10	-0.46	-1.06	**
Fatty acid	enoyl coenzyme A hydratase 1	AA926032	-0.04	-0.23	-1.07	**
Fatty acid	Fatty acid binding protein 1, liver	AI072074	-0.25	-0.31	-1.16	***
Fatty acid	Fatty acid binding protein 1, liver	AI072074	-0.05	-0.28	-1.19	***
Fatty acid	carnitine palmitoyltransferase 1	AA875270	-0.13	-0.06	-1.25	***
Fatty acid	fatty acid Coenzyme A ligase, longchain 2	AA926010	-0.03	-0.25	-1.56	***
Glucose	glucose-6-phosphatase, (G6pc)	AA964628	-0.60	-0.46	-1.58	**
Glucose	alanine-glyoxylate aminotransferase	AI029012	-0.16	-0.17	-0.88	**
Glycolysis	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	AA924111	0.08	0.48 **	1.07	***
Immuno.	melanoma antigen, family D, 1	AA925628	-0.03	-0.26	-0.76	***
PKC;	14-3-3 Protein, Ywhaz	AA858662	-0.40	-0.68 **	-0.98	**
Prot.synth.	High sim to eIF-5A (eIF-4D) [Mouse]	AA874949	0.07	0.45	0.95	**
Prot.synth.	High sim to eEF-1B gamma [Mouse]	AA818663	0.11	0.45	0.74	**
Proteolysis	proteasome beta type 6	AA925552	0.39 **	-0.17	1.68	**
Proteolysis	plasma glutamate carboxypeptidase	AI145442	0.66	1.06	1.62	**
Proteolysis	polyubiquitin	AA875068	-0.12	0.12	0.91	**
Proteolysis	complement component 1, s	AA965204	-0.26	-0.56	-1.32	***
Proteolysis	cathepsin S	AA925933	-0.28	-0.34	-1.33	**
Ribosome	ribosomal protein L7	AA956776	0.25	0.49 **	0.87	***
Ribosome	ribosomal protein S2	AA858477	0.17	0.51	0.82	**
Ribosome	ribosomal protein L41	AI029242	0.28	0.41	0.71	**
Ribosome	ribosomal protein L21	AA819765	-0.34	-0.37	-1.24	**
Structure	High sim to S11222 actin gamma	AA964496	0.53	0.52	2.73	***
Structure?	Weak sim to pervin [Rat]	AA964725	0.48	0.43	2.43	**
Structure	High sim to alpha-tubulin [Rat]	AI705683	0.27	0.33	1.48	***
Structure	actin, beta	AA859846	0.24	0.34	1.28	**
Structure	keratin complex 1, acidic, gene 18	AI072634	0.22	0.90 **	1.19	**
Structure	alpha-tubulin	AA957078	-0.23	-0.17	0.81	***
Structure	cysteine-rich protein 3	AA925907	-0.21	-0.53	-1.31	**
Sign.transd	GTP-binding protein (ral A)	AA900726	0.06	0.33	0.81	**
Sign.transd	asialoglycoprotein receptor 2	AA997920	-0.32	-0.37 **	-1.11	**
Sign.transd.	synaptojanin 2	AA963164	-0.09	-0.32	-1.60	***
heme met	hemoglobin beta chain complex	AA818084	0.25	-0.03	2.14	**
heme	hemoglobin, alpha 1	AA819784	0.38	0.10	2.06	**
Transport	sodium channel, voltage-gated, type 6, alpha polypeptide	AA925248	-0.05	-0.32	-0.89	**
Other	homer, neuronal immediate early gene, 2	AI059976	0.73	1.38 **	0.82	**
Other	Weak sim to Y37E11B.5.p	AI072198	0.01	0.03	0.79	**
Other	sim to apoptotic protease activ. factor 1	AA818945	0.29	0.29	0.75	**
Other	DD6G4-2	AA924781	-0.18	-0.21	-0.71	***
Other	lumican	AA866389	-0.17	-0.17	-0.74	**
Other	dimethylglycine dehydrogenase	AA997856	-0.19	-0.28	-0.75	**

Other	Weak sim to Arginase II, mitoch.[Rat]	AI028979	0.02	0.04	-0.75	**
Other	kynurenine aminotransferase 2	AA998607	-0.07	-0.29	-0.75	**
Other	ras-related GTP-binding protein 4b	AA818114	-0.13	-0.15	-0.77	**
Other	carboxylesterase 3	AA955115	-0.22	-0.35	-0.85	**
Other	DD6G4-2	AA923983	-0.10	-0.20	-0.88	***
Other	guanylate cyclase 1, soluble, beta 3	AA955420	-0.30	-0.56	-0.97	**

Table III. 3. *Gene expression changes 48 h after bromobenzene dosage*

The subset of genes that were significantly ($p < 0.01$) differentially expressed after 48 h in the high BB dosed rats compared to the controls. Genes were categorised according to biological mechanism or pathways. The log (base 2) average fold change and the p-value for the three dose levels are listed. A threshold was chosen for display reasons which was a log (base2) fold change > 0.7 or < -0.7 in any of the dose groups compared to the controls. Statistical level of significance: **: $p < 0.01$, ***: $p < 0.001$. ACC#: Genbank Accession number, metab: metabolism

Category	*Gene name*	*ACC #*	*low*	*mid*	*high*	
Acute phase	Orosomucoid 1	AI029162	0.33	0.02	1.98	***
Acute phase	pancreatic secretory trypsin inhibitor type II	AA858673	-0.16	0.15	1.84	***
Acute phase	ferritin, heavy polypeptide 1	AA818441	-0.33	-0.01	1.19	**
Acute phase	osteonectin	AA957962	-0.05	0.17	0.91	**
Acute phase	apolipoprotein A-I (apoA-I)	AA964044	0.30	0.49	0.83	**
Acute phase	*Weak sim to Ficolin 2 precursor*	AA859022	0.14	-0.22	-0.72	***
Acute phase	fibrinogen-like protein 1	AI044677	-0.38	-0.43	-0.88	**
Acute phase	pre-alpha-inhibitor, heavy chain 3	AI030246	-0.19	-0.47	-0.79	**
Acute phase	Weakly sim. to serum amyloid P-Component	AA819101	0.00	-0.21	-0.93	**
Acute phase	alpha(1)-inhibitor 3, variant I	AA817963	-0.30	-0.31	-1.69	***
Acute phase	alpha(1)-inhibitor 3, variant I	AA817963	-0.17	-0.38	-1.73	**
Acute phase	Serine protease inhibitor	AA901050	0.12	0.17	-2.47	***
Ox.stress	Heme oxygenase (HO-1)	AA874884	0.16	0.13	1.07	**
Ox.stress	peroxiredoxin 1	AA875245	0.08	-0.02	1.04	***
Ox.stress	peroxiredoxin 1	AA875245	0.12	0.00	1.02	***
Ox.stress	Heme oxygenase (HO-1)	AA874884	-0.28	-0.01	0.83	**
Aminoacid	ornithine aminotransferase	AA818680	0.00	-0.18	-0.82	***
Aminoacid	argininosuccinate lyase	AA818673	0.13	-0.15	-1.40	***
Cysteine.	cytosolic cysteine dioxygenase 1	AA818579	-0.28	-0.23	-1.14	***
Drug;GSH	glutathione S-transferase, alpha 1 (GSTA)	AA818339	0.16	0.53	2.31	**
Drug	epoxide hydrolase 1 (mEH)	AA900551	0.09	0.10	2.13	**
Drug	High sim to RAKb [Rat] (aldo-keto reductase)	AA817695	0.02	0.20	1.87	***
Drug;GSH	glutathione S-transferase, alpha 1 (GSTA)	AA818339	0.17	0.51	1.71	**
Drug	aflatoxin B1 aldehyde reductase (AFAR)	AA923966	0.14	0.13	1.46	**
Drug;GSH?	High sim to GSTA 8 [Rat]	AI059894	0.40	0.44	** 0.75	**
Drug	aldehyde dehydrogenase family 3, A1	AA866268	-0.09	0.03	0.74	**
Drug	aldo-keto reductase family 1, member A1	AA875038	-0.03	0.20	0.71	***
Drug	sulfotransferase family, cytosolic, 2A, 1	AA819605	-0.10	-0.19	-0.82	**
Drug	sulfotransferase, hydroxysteroid preferring 2	AA818024	0.06	-0.36	-0.82	**
Drug	arachidonic acid epoxygenase;Cyp2C23	AA819756	-0.16	0.15	-0.87	**
Drug	cytochrome *P450 2d18* (Cyp2D18)	AA997886	0.28	-0.19	-0.94	**
Drug	cytochrome P450 4A3 (Cyp4A3)	AA924591	-0.14	-0.01	-0.97	***
Drug	cytochrome P450, 4a10 (Cyp4A10)	AA956787	-0.15	0.00	-1.45	**
Drug	cytochrome P450, subfamily 2E, (Cyp2E1)	AA818896	-0.04	0.19	-2.25	***
Fatty acid	delta3, delta2-enoyl-CoA isomerase	AA997009	-0.24	-0.07	-0.77	**
Fatty acid	dodecenoyl-coenzyme A delta isomerase	AA965078	-0.33	0.07	-0.92	**
Fatty acid	*fatty acid binding protein 4*	AA925091	0.27	0.20	0.79	**

Glycolysis	Glyceraldehyde-3-phosphate dehydrogenase	AA924111	0.17	-0.21	1.03	***
signal transd.	protein kinase C, delta	AI044238	0.38	0.38	0.85	**
signal transd.	14-3-3 protein, Ywhaz	AA858662	0.23	0.07	-0.92	***
prot.synthesis	High sim to eEF-1B gamma [Mouse]	AA818663	0.10	0.02	0.77	**
proteolysis	carboxypeptidase A1	AA956202	1.20	0.74	1.08	**
proteolysis?	polyubiquitin	AA875068	0.27	0.44	0.88	**
Ribosome	ribosomal protein S2	AA819544	0.01	0.27	1.26	**
Ribosome	ribosomal protein L41	AI029242	0.50	0.56	1.22	**
Ribosome	ribosomal protein S27a	AA899215	0.38	0.38	1.04	**
Ribosome	peptidyl arginine deiminase, type 2	AA818640	0.17	0.54	1.03	**
Ribosome	High sim to 40S ribosomal protein S16 [Rat]	AA860041	0.17	0.05	0.99	**
Ribosome	ribosomal protein L21	AA819404	-0.08	-0.04	0.99	**
Ribosome	High sim to 60S ribosomal protein L37a [Rat]	AA900050	0.29	0.32	0.96	**
Ribosome	ribosomal protein S23	AA900188	0.22	0.19	0.95	**
Ribosome	High sim to 60S ribosomal protein L38 [Rat]	AA858971	0.01	0.04	0.93	**
Ribosome	Mod.sim to human exonuclease	AA899560	0.01	0.23	0.93	**
Ribosome	ribosomal protein L6	AA924912	0.44	0.22	0.90	**
Ribosome	ribosomal protein L13	AA859756	0.22	0.24	0.90	**
Ribosome	ribosomal protein S8	AA874997	0.11	0.09	0.86	**
Ribosome	ribosomal protein L4	AA817847	0.19	0.21	0.84	**
Ribosome	ribosomal protein S26	AA900527	0.07	0.34	0.84	**
Ribosome	NADH dehydrogenase (ubiquinone) 1 alpha 5	AA874885	0.26	0.12	0.82	**
Ribosome	DD6G4-2	AI711101	-0.20	0.05	0.81	***
Ribosome	ribosomal protein L7 mRNA, 5' end	AA956776	0.39	0.22	0.80	**
Ribosome	ribosomal protein S2	AA858477	0.06	0.14	0.79	**
Ribosome	ribosomal protein L35	AA925200	0.40	0.37	0.77	**
Ribosome	ribosomal protein S11	AA818442	-0.01	0.07	0.74	**
Ribosome	ribosomal protein L21	AA819765	-0.10	-0.01	-1.06	***
Structure	actin, beta	AA859846	0.11	0.04	1.32	**
Structure	alpha-tubulin	AA957078	-0.11	-0.14	1.30	***
Structure	thymosin beta-4	AA819102	-0.01	0.04	1.22	**
Structure	smooth muscle alpha-actin	AA819435	0.07	0.13	0.81	**
Structure	cortactin isoform B	AI069969	-0.10	0.21	0.78	**
Structure	dynein, cytoplasmic, light chain 1	AA962992	0.13	0.01	0.73	**
Structure	calpactin I heavy chain	AA859635	-0.41	0.19	0.72	**
Sign.transd.	casein kinase II beta subunit	AA997175	0.07	-0.02	2.05	**
Sign.transd.	GTP-binding protein (ral A)	AA900726	0.04	0.13	1.08	***
Sign.transd.	pyruvate kinase, muscle	AA818951	0.02	0.28	0.87	**
Sign.transd.	guanine nucleotide binding protein	AA859083	0.02	0.39	0.85	**
Sign.transd.	core promoter element binding protein	AA900368	-0.36	-0.26	-0.70	***
Sign.transd.	asialoglycoprotein receptor 2	AA997920	-0.01	0.09	-0.84	**
Sign.transd.	Sim.to 3',5'-cyclic AMP phosphodiesterase	AA858930	0.06	-0.22	-0.85	***
Sign.transd.	gap junction membrane channel beta 5	AI044985	0.20	0.13	-0.93	**
Sign.transd.	EST, phosphatase?	AA926359	0.24	0.16	-1.28	**
Transport	ESTs	AI043642	0.06	0.26	0.80	***
Transport	sim to myo-inositol 1-phosphate synthase A1	AA933167	0.03	0.17	0.79	**
Transport	sodium channel, voltage-gated, type 6, alpha	AA925248	-0.43	0.05	-0.70	**
Transport	ATPase, H+ transporting, lysosomal	AA858959	-0.22	-0.27	-0.71	**
Transport	solute carrier family 29, member 2	AA859360	-0.22	-0.21	-0.71	**
Other	secreted phosphoprotein 1	AA964431	0.18	0.17	1.24	**
Other	vimentin	AA859385	-0.04	0.03	1.10	***

Other	Weak sim to interferon,alpha-inducible prot.	AA819034	-0.03	0.16	1.09	**
Other	S100 calcium binding protein A10 (calpactin)	AA900235	0.04	0.21	1.04	***
Other	clathrin light chain (LCB2)	AI579822	0.12	0.18	0.96	**
Other	sim to HUMAN Small membrane protein 1	AA818246	-0.02	-0.13	0.91	**
Other	serine (or cysteine) proteinase inhibitor, B5	AA899906	0.15	0.00	0.83	**
Other	secretoglobin, family 1A, member 1	AA817804	-0.06	0.60	0.79	**
Other	stathmin 1	AA957519	0.14	0.11	0.72	**
Other	Seminal vesicle protein, secretion 2	AA900854	0.01	0.16	0.70	**
Other	retinol dehydrogenase type III	AA866390	-0.24	-0.15	-0.74	***
Other	lumican	AA866389	-0.23	-0.19	-0.76	**
Other	High sim to GENE 33 POLYPEPTIDE [Rat]	AA997280	0.15	0.16	-0.82	**
Other	retinol dehydrogenase type III	AA866390	-0.27	-0.27	-0.85	**
Other	sim to alcohol dehydrogenase	AA818154	-0.60	-0.34	-0.87	***
Other	carboxylesterase 3	AA955115	-0.27	-0.22	-0.95	***
Other	guanylate cyclase 1, soluble, beta 3	AA955420	0.04	0.16	-1.13	**

Appendix of Chapter VI

Table VI.2E. Genes affected by trichloroethylene, related to various biological processes. The level of statistical significance (Student's T-tests) is indicated to the mean log(2) transformed fold changes, and listed in the last column for the ANOVA **(A)** (*: $p < 0.02$, **: $p < 0.01$, ***: $p<0.001$) . Genes in boldface appeared in the list of 100 most significantly up- or downregulated genes in the PCDA analysis.

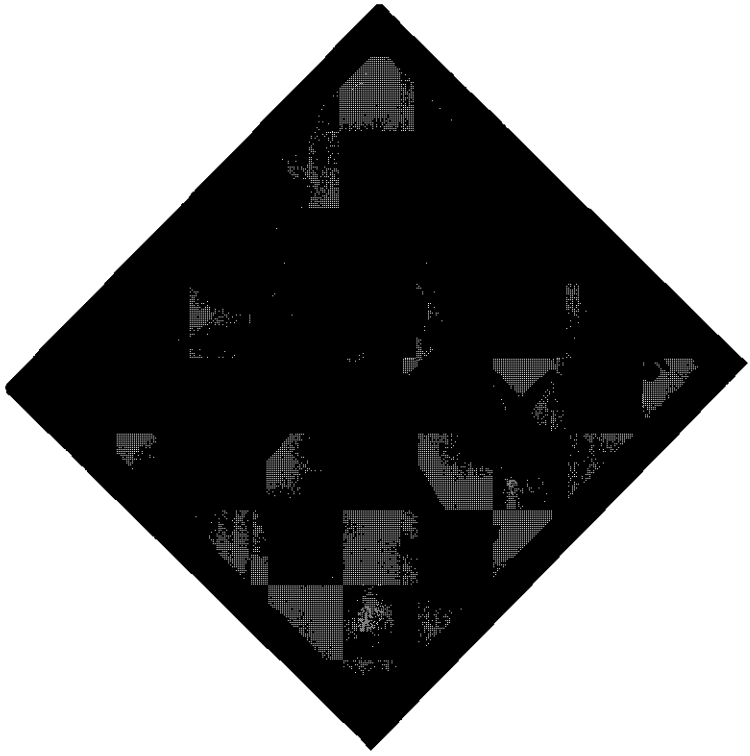
Class	Acc#	Symbol	GeneName	Low	Mid	High	A
acute phase	NM_017096	Crp	**C-reactive protein**	0.70	0.37*	0.87**	**
acute phase	NM_012657	Spin2b	**Serine protease inhibitor**	0.32	0.35	0.70**	**
acute phase	NM_017170	Sap	Serum amyloid P-component	0.44*	0.66**	0.59*	**
acute phase	NM_019301	Crry	Complement receptor related	-0.1	-0.07	-0.30**	**
acute phase	NM_024157	Cfi	**Complement factor I**	0.12	-0.28**	-0.56*	***
acute phase	NM_017351	Paihc3	**Pre-alpha-inhibitor, heavy chain 3**	-0.6**	-0.42**	-0.69**	***
acute phase	NM_031531	Spin2c	**Serine protease inhibitor**	-0.7**	-0.46**	-0.82**	***
amino acids	NM_017074	Cth	**CTL target antigen**	0.33	0.07	0.70**	**
amino acids	NM_013078	Otc	Ornithine carbamoyltransferase	0.59*	0.32***	0.26	***
amino acids	NM_017084	Gnmt	Glycine methyltransferase	0.53**	0.39	0.14	**
amino acids	NM_022403	Tdo2	Tryptophan 2,3-dioxygenase	0.53**	0.24*	-0.05	***
amino acids	J05499	Ga	Liver mitochondrial glutaminase	-0.4**	-0.35**	-0.26	*
amino acids	NM_021750	Csad	**Cysteine-sulfinate decarboxylase**	-0.2	-0.08	-0.56**	**
amino acids	NM_019386	Tgm2	**Tissue-type transglutaminase**	-0.7**	-0.22	-0.56*	**
amino acids	NM_031012	Anpep	**Alanyl aminopeptidase**	-0.5*	-0.44**	-0.81**	**
amino acids	NM_012668	Tat	**Tyrosine aminotransferase**	0.20	-0.33	-0.92**	***
amino acids	NM_022521	Oat	**Ornithine aminotransferase**	0.10	-0.83***	-1.53***	***
Apoptosis	AI599423		**sim. to GADD45,.**	0.82***	0.31	0.77***	***
apoptosis	BG671464		**sim.to programmed cell death 10**	0.85**	0.65**	0.70***	***
apoptosis	M15481	Igf1	**Insulin-like growth factor 1**	-0.0	-0.09	-0.60**	*
cancer?	X78606	Rab28	RAB28, member RAS oncogene	0.05	0.21	0.32**	*
cancer?	NM_012646	Rt1-n1	RT1 class Ib, H2-TL-like, grc region	-0.7	-0.37*	-0.76**	**
cell_adhes	NM_012811	Mfge8	O-acetyltransferase Milk fat globule	-0.5**	-0.06	-0.56**	***
cell_adhes	NM_019375		**Septin 3**	0.16	0.35	-1.00**	***
cell_adhes	X78997	Cdh17	**Cadherin 17**	-0.4**	-0.64***	-2.39***	***
defense	NM_053469	Hamp	hepcidin antimicrobial peptide	0.72*	-0.41	-0.08	***
defense	NM_012512	B2m	**Beta-2-microglobulin**	0.62	0.48*	0.55*	*
DNA_repair	X54862	Mgmt	**O6-methylguanine-DNA**	0.12	0.78**	0.67	**

			methyltranferase				
DNA_repair	NM_012699	Dnajb9	DnaJ homolog, subfamily b, 9	0.32	0.03	-0.41	**
e-_transp	NM_012839	Cycs	Cytochrome c, somatic	0.68**	0.32*	0.34	**
glucose?	NM_022268	Pygl	**Liver glycogen phosphorylase**	-0.0	-0.48	-0.80**	**
glycolysis	M76591	Pgam1	Phosphoglycerate mutase 1	0.08	0.31	0.51*	*
glycolysis	NM_013098	G6pc	**Glucose-6-phosphatase (G6pc)**	-1.0***	-0.98**	-1.59***	***
glycolysis	NM_012624	Pklr	**Pyruvate kinase, liver and RBC**	-0.5	-1.14*	-1.35*	*
glycolysis?	NM_031749	Gcs1	**Glucosidase 1**	-0.6*	-0.14	-0.55**	**
heme	X05080	Hbb	Hemoglobin, beta	0.81**	0.88**	0.53*	***
heme	NM_013096	Hba1	Hemoglobin, alpha 1	-0.1	0.31**	-0.02	*
heme	AF307840	Ncb5or	Flavohemoprotein b5+b5R	0.19	0.34*	0.36*	*
hemopoies.	NM_024385	Hhex	Hematopoietically expressed homeobox	-0.7	0.01	-0.95**	**
immunor.	X56596		**MHC class II antigen RT1.B-1 beta**	-0.7***	-0.41*	-0.55	*
immunor.	AF029240		**MHC class Ib RT1.S3 (RT1.S3)**	0.01	-0.47*	-0.78**	***
sign_transd	AF057025	Tlr4	Toll-like receptor 4	0.46	0.71	0.53	**
sign_transd	NM_022392		Growth response protein (CL-6)	0.02	-0.66*	0.28	**
sign_transd	U61373	F2rl1	Proteinase-activated receptor-2,	0.01	0.51	0.17	**
sign_transd	NM_021764		Protein kinase C-binding Beta15	0.02	0.37*	0.11	**
sign_transd	X73371	Fcgr2b	Fc gamma receptor	0.73***	0.43	0.10	**
sign_transd	AB027562	Sh3bp5	SH3-domain binding protein 5	-0.0	0.17	-0.30	**
sign_transd	NM_012715	Adm	Adrenomedullin	-0.0	0.11	-0.35**	***
sign_transd	NM_031822	Ncoa2	Nuclear receptor coactivator 2	-0.6	-0.43	-0.42	**
sign_transd	NM_021676	Shank3	SH3/ankyrin domain 3	-0.5**	-0.28	-0.46	*
sign_transd	M96548	Znf354a	Zinc finger protein 354A	0.17	0.44**	-0.47	**
sign_transd	NM_016991	Adra1b	**Adrenergic, alpha 1B, receptor**	-0.6	-0.20	-0.52	**
sign_transd	NM_019249	Ptprf	**Protein tyrosine phosphatase, receptor type, F**	-0.4	-0.74***	-0.64**	***
sign_transd	AY014898	Ipmk	**Inositol polyphosphate multikinase**	-0.5*	-0.46**	-0.67*	**
sign_transd	AB030238		**Sim. to hepatocarcinogenesis-related transc.factor**	-0.3	-0.34	-1.01***	***
sign_transd	NM_019232	Sgk	**Serum/glucocorticoid regulated kinase**	-0.9	-0.86*	-1.02***	**
steroid	NM_031682	Hsd17b 10	**Hydroxysteroid (17-beta) dehydrogenase 10**	0.42**	0.47**	0.55***	***
steroid	J05035	Srd5a1	Steroid 5 alpha-reductase 1	-0.3	0.34	0.43	*
stress	BG668317	Hspca	**heat shock protein 1, alpha**	0.77*	0.80***	0.71	**
stress	NM_024351	Hspa8	**Heat shock cognate protein 70**	0.08	0.07	-0.61***	***
structure	M15883	Cltb	**Clathrin, light polypeptide (Lcb)**	0.15	0.66**	0.62**	***
structure	NM_019167	Spnb3	Beta-spectrin 3	-0.6*	-0.29	0.11	**
structure	X70369	Col3a1	Collagen, type III, alpha 1	-0.2	-0.54**	-0.54**	**
structure	NM_031136	Tmsb4x	Thymosin beta-4	0.50	-0.19	-0.60	**
transport	NM_022388	Fxyd4	FXYDdomain-containing ion transport regulator 4	0.15	0.52*	0.60*	**
transport	M13979	Slc2a1	Solute carrier family 2,member 1	0.60**	0.21	0.57**	**
transport	NM_019158	Aqp8	Aquaporin 8	-0.1	0.48*	0.50	*
transport	L19927	Atp5c1	ATP synthase, H+ transporting, mit. F1 complex, gamma 1	0.56*	0.26*	0.41	**
transport	Y12635	Atp6b2	ATPase, H+ transporting, lysosomal	0.42**	0.14	0.19	**
transport	AB006450	Timm17 a	Translocator of inner mitoch membrane 17a	-0.3	0.24	0.08	**
transport	NM_019186	Arl4	ADP-ribosylation-like 4	0.17	0.48***	-0.26	**
transport	NM_145677	Pcscl	peroxisomal Ca-dependent solute carrier	0.03	-0.24	-0.42	**
transport	NM_031746	Slc13a2	Solute carrier family 13, member 2	-0.6**	-0.08	-0.42*	**

transport	NM_012833	Abcc2	ATP-binding cassette, sub-family C (CFTR/MRP), member 2	0.30	-0.06	-0.43	**
transport	NM_031725	Scamp4	**Secretory carrier membrane protein 4**	-0.5	0.07	-0.58	*
transport	NM_022287	Slc26a1	**Sulfate anion transporter**	-0.2*	-0.33**	-0.63**	***
transport	AF019628	Abcc9	**ABC transporter C (CFTR/MRP) 9**	-0.1	-0.17	-0.73*	**
unknown	BG666041		**sim. to HSPC288 (LOC299207)**	0.74**	0.58*	0.81	**
unknown	BE112955		sim. to MGC4614 (LOC294294)	0.56	0.64**	0.67**	*
other	NM_017132	Rcn2	**Reticulocalbin 2**	0.19	0.91*	1.55**	***
other	D00569	Decr1	**2,4-dienoyl CoA reductase 1, mit.**	0.75**	0.92***	1.27***	***
other	NM_012600	Me1	**Malic enzyme 1, soluble**	0.47*	0.70**	1.27***	***
other	AF062389		**Kidney-specific protein (KS)**	0.24	0.54	1.11**	***
other	L46593		**sim. to cornifin alpha (SPRR1)**	0.56	1.06*	1.02*	*
other	D28560	Enpp2	**Ectonucleotide pyrophosphatase**	0.51	0.59***	0.90**	**
other	NM_022513		**Dopa/tyrosine sulfotransferase**	0.78***	0.68**	0.81*	***
other	NM_147138	Sip30	**SNAP25 interacting protein 30**	0.56	0.26	0.79*	**
other	NM_144743		***carboxylesterase isoenzyme***	0.93***	0.09	0.79	***
other	D85435	Prkcdbp	**PKC-delta binding protein**	0.28	0.32	0.76**	**
other	NM_130400	Dhfr	Dihydrofolate reductase 1 (active)	0.77**	0.35	0.58	**
other	BG671436		sim. to Ubiquinol-cytochrome C reductase complex core protein 2	0.92	0.55**	0.48	*
other	NM_013010	Prkag1	Protein kinase, AMP-activated, gamma 1 non-catalytic	0.30	0.62**	0.45	**
other	X72759		cox VIa pseudogene (liver)	0.32	0.26**	0.45	*
other	NM_138839	Vmp1	vacuole Membrane Protein 1	0.53	0.68*	0.44	**
other	AF281632	Sybl1	Synaptobrevin-like 1	0.49	0.58	0.36	**
other	M22030		**sim. to electron transfer flavoprotein alpha**	0.68**	0.27	0.35	**
other	NM_022629	Bbox	Gamma-butyrobetaine hydroxylase	0.57**	0.18	0.33	*
other	M22756	Ndufv2	**24-kDa subunit of mitochondrial NADH dehydrogenase**	0.13	0.52**	0.29	*
other	AW141131		sim. to CGI-35 protein (LOC299198)	0.01	0.67**	0.27	***
other	X77236		Protein phosphatase V	0.43**	0.45**	0.27	**
other	AF151784	Yme1l1	YME1 (S.cerevisiae)-like 1	0.44**	0.56**	0.26	***
other	NM_013003	Pemt	Phosphatidylethanolamine N-methyltransferase	-0.2	0.32	0.24	**
other	AF526268	Pgr1	T-cell activation protein-related protein	0.41	0.09	0.23**	**
other	NM_020082	Rnase4	Ribonuclease, RNase A family 4	0.77	-0.06	0.13	**
other	AJ301634	Sca10	Spinocerebellar ataxia 10 homolog (human)	-0.3	0.26	0.11	**
other	M36708	Ass	Arginosuccinate synthetase 1	0.39	-0.42	0.07	**
other	BG668391		sim. to NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 7	-0.4**	0.09	-0.02	*
other	M83680	Rab14	GTPase Rab14	-0.2**	0.26	-0.07	**
other	M29295	Snrpb	Small nuclear ribonucleoprotein polypeptides B and B1	-0.5***	-0.03	-0.12	**
other	AF205717	Tm4sf4	Transmembrane 4 member 4	0.17	-0.29	-0.18	**
other	NM_019201	Ctbp1	C-terminal binding protein 1	-0.3	0.15	-0.28	**
other	X53003	Acac	acetyl-coenzyme A carboxylase	-0.5**	-0.39**	-0.33*	**
other	NM_021576	Nt5	5 nucleotidase	0.13	-0.26	-0.38*	**
other	NM_022532	Araf1	A-raf	-0.4	-0.02	-0.38**	**
other	NM_021744	Cd14	CD14 antigen	-0.5**	-0.19	-0.46*	**
other	NM_012976	Lgals5	**Lectin, galactose binding 5**	-0.4	-0.31**	-0.47**	**
other	Y13413	Apbb3	Amyloid beta (A4) precursor protein-binding, family B, member 3	-0.4	-0.14	-0.50*	**
other	D50564	Mpst	Mercaptopyruvate sulfurtransferase	-0.7**	-0.20	-0.52*	***
other	D25233	Rb1	Retinoblastoma 1	-0.5	-0.35	-0.55	**

other	NM_013086	Crem	CAMP responsive element modulator	0.22	0.05	-0.55**	***
other	NM_024143		Bile acid CoA ligase	-0.0	-0.39	-0.57*	*
other	AJ242926	Fetub	Fetuin beta	-0.6	-0.51***	-0.58*	**
other	NM_019384		CTD-binding SR-like rA1	-0.4**	-0.26	-0.59**	**
other	Y17328	Crym	**Crystallin, mu**	0.04	0.15	-0.60	**
other	X76456		**alpha albumin**	-0.0	-0.24*	-0.61**	**
other	AF281635	Znf22	Zinc finger protein 22 (KOX 15)	-0.6	-0.25	-0.63**	**
other	AF168795	Slfn4	Schlafen 4	-0.6**	-0.59	-0.66**	**
other	NM_032082	Hao3	**Hydroxyacid oxidase 3**	0.33	0.08	-0.72***	***
other	NM_017343	Mrlcb	**Myosin regulatory light chain**	-0.8**	-0.29	-0.74	**
other	NM_021594		**ERM-binding phosphoprotein**	-0.4**	-0.30	-0.87**	***
other	NM_013069	Cd74	**CD74 antigen**	-0.5	-0.80**	-0.97**	**
other	AF072439	Zfp37	**Zinc finger protein 37**	-0.3	-0.19	-1.00***	***
other	NM_019292	Ca3	**Carbonic anhydrase 3**	0.09	-0.38	-1.21**	**
other	NM_147215	Obp3	**alpha-2u globulin PGCL4**	0.35	0.42	-1.30***	***

Samenvatting



Toxicogenomics

Toepassingen van de nieuwe "functional genomics" technologieën in de toxicologie

De studies in dit proefschrift zijn uitgevoerd om te bepalen wat de waarde is van het toepassen van toxicogenomics methoden in de toxicologie.
Wat is toxicogenomics ?
Het woord toxicogenomics is een samentrekking van *toxicologie* en *genomics*, en beschrijft de toepassing in de toxicologie van nieuwe technologieën gebaseerd op kennis van het *genoom* (de genen). In de volgende paragrafen zal uitgelegd worden wat toxicologie is en waarvoor de nieuwe methoden kunnen dienen.

Toxicologie

De toxicologie bestudeert de effecten van giftige stoffen uit de omgeving op het lichaam. Er zijn methoden ontwikkeld om giftige eigenschappen van stoffen aan te tonen, bijvoorbeeld door gebruik van proefdieren, maar ook *in vitro* testen (in een reageerbuis). De routinematig toegepaste testen geven meestal aan dat er nadelige effecten van blootstelling optreden, maar niet hoe de toxiciteit van stoffen verklaard wordt. Er wordt bepaald bij welke blootstelling het geen-nadelig-effect-niveau van een stof wordt bereikt. Het onderliggende mechanisme van toxiciteit dat tot de schade leidt is echter vaak nog niet bekend.
Om bovenstaande redenen, maar ook om dierproeven te voorkomen, zijn toxicologen op zoek naar nieuwe methoden om toxiciteit aan te tonen, te verklaren en liever nog, te voorspellen. Er wordt al steeds nauwkeuriger bestudeerd welke veranderingen er optreden in organen, weefsels, en cellen van blootgestelde dieren of *in vitro* modellen. De pathologie maakt veelvuldig gebruik van microscopie. Daarnaast zijn er klinisch-chemische technieken om gehaltes van enzymen en metabolieten te bepalen (ook wel toxiciteitsmarkers genoemd). Deze kunnen een aanwijzing geven voor het optreden van schade. Verder behoren biochemische bepalingen en hematologie (bloedonderzoek) nog tot de conventionele technieken.
Moleculair-biologische technieken maken metingen mogelijk aan cellulaire eiwitten, DNA (chromosomen en genen) en daaraan verwant RNA. De recent ontwikkelde 'functional genomics' technologieën passen de moleculaire technieken toe op veel grotere schaal.

"Functional genomics" technologieën

Een zeer belangrijke vooruitgang in de wetenschap was onlangs het ontcijferen van de samenstelling van het menselijk genoom, bestaande uit ongeveer 30000 verschillende genen. De genen bevatten de codes voor het produceren van de duizenden verschillende eiwitten in de cellen. Figuur I.2 in hoofdstuk I geeft schematisch weer hoe functionele eiwitten gevormd worden met de genen als informatiebron.

In elke cel van het lichaam zijn alle 30000 genen aanwezig, als DNA in de chromosomen. Ze bevatten de informatie nodig voor de aanmaak van eiwitten die dienen als enzymen en bouwstoffen in de cel. De genen zelf veranderen niet of nauwelijks. Wel kan de activiteit (expressie) van de genen veranderen. Die genexpressie bestaat uit het kopiëren van de informatie van het gen naar informatiedragers, de boodschapper RNA's (mRNA's). De boodschappers vormen als het ware een mal voor de samenstelling van eiwitten uit afzonderlijke aminozuren. De eiwitten zijn uiteindelijk verantwoordelijk voor de structuur en de biochemische processen in de cellen, en daarmee ook voor de veranderingen in weefsels en uiteindelijk ook het lichaam.

Toxicogenomics

De "functional genomics" technologieën *genomics, transcriptomics, proteomics en metabolomics bestuderen* respectievelijk de samenstelling van het *genoom*, de *expressie* daarvan, de *eiwitten* en de *metabolieten* (zie tabel I.1). Verschillende technieken zijn recent ontwikkeld om de verschillende onderdelen van de cel te meten.

Transcriptomics – De uitvinding van de DNA microarray of DNA chip maakt het mogelijk van duizenden genen tegelijk de expressie of activiteit te meten. Een DNA microarray is een microscoopglaasje met daarop duizenden verschillende DNA codes die elk een ander gen vertegenwoordigen. Het mRNA gehalte is een maat voor de expressie van een gen en voorspelt veranderingen in gehaltes van eiwitten die een rol spelen in processen in een cel.
Het mRNA wordt geïsoleerd uit blootgesteld en uit onbehandeld weefsel. Door van beide monsters alle mRNA's samen op de DNA microarray te brengen kan per gen ineens uitgelezen worden wat de verhouding is in expressie van duizenden verschillende genen in blootgesteld t.o.v onbehandeld weefsel. Omdat mRNA moleculen ook wel transcripten van genen genoemd worden, heet deze methode transcriptomics. Het meten van genexpressie met DNA microarrays is schematisch weergegeven in Figuur I.3a.

Proteomics – Metingen aan eiwitten (proteïnen vandaar proteomics) kunnen meer inzicht opleveren dan genexpressiemetingen. Het zijn nl. de eiwitten die de meeste functies in de cel vervullen. (zie ook figuur I.1). Daarnaast kunnen de metingen bijvoorbeeld aantonen of de eiwitten actief of inactief zijn. Het meten van duizenden verschillende eiwitten wordt echter bemoeilijkt doordat eiwitten allemaal verschillende eigenschappen bezitten. Voorts is de identificatie van de gemeten eiwitten nog een beperkende factor.

Metabolomics – Het meten van gehaltes van alle kleine moleculen (metabolieten) in een cel weefsel, of lichaamssappen is het doel van de metabolomics technologie. Deze gehaltes kunnen bevestigen dat eiwitten (enzymen) bepaalde omzettingen hebben verricht (zie ook figuur I.1). Ophoping of juist tekorten aan bepaalde metabolieten kunnen duiden op veranderingen in het lichaam.
Het is op dit moment nog lastig om van alle gemeten moleculen de identiteit vast te stellen. Een groot voordeel van metabolomics is dat ook monsters geanalyseerd kunnen worden die gemakkelijk verzameld kunnen worden zoals bloed en urine.

Toxicologie op molecuulniveau

Het lichaam beschikt over inventieve mechanismen om gifstoffen af te breken en uit te scheiden. Zo kunnen toxische stoffen (zoals alcohol, benzeen, broombenzeen) bepaalde genen stimuleren tot aanmaak van gif afbrekende enzymen. Een voorbeeld hiervan is de toename van de aanwezigheid van alcohol afbrekende enzymen na inname van alcohol. Dit is een respons die zijn oorsprong vindt in verhoogde expressie van genen die aanzetten tot aanmaak van alcohol dehydrogenase en Cyp2e1.
Ook kan een gif processen in de cel zoals de celdeling of de aanmaak van cholesterol verstoren. Dat gebeurt onder andere als het gif ervoor zorgt dat genen betrokken bij die processen meer of minder actief worden.
Blootstelling aan gifstoffen kan dus de genexpressie beïnvloeden. Dit kan vervolgens leiden tot verandering in het functioneren van de cel, het orgaan of het lichaam. Hierdoor kunnen genexpressieveranderingen in een vroeg stadium een indicatie geven voor effecten die op

dat moment nog niet aantoonbaar zijn met pathologie, gebruik makend van microscopie. Ook kunnen de effecten wellicht al optreden bij lage blootstelling die nog geen uiterlijk veranderingen veroorzaken. Dat is belangrijk omdat in werkelijkheid vaak het risico van langdurige blootstelling aan lage concentraties beoordeeld moet worden.

Transcriptomics bepalingen van de expressie van duizenden genen tegelijk (m.b.v. de DNA microarray) kunnen dus gevoeliger zijn in het aantonen van verstoringen door gifstoffen dan de gangbare technieken. Ook is aangetoond dat het op grote schaal meten van metabolieten in bloed of urine m.b.v. metabolomics al bij lage doseringen verschillen kan aantonen.

Misschien wel het allerbelangrijkste is dat toxicogenomics de mogelijkheid biedt inzicht te krijgen hoe en waarom stoffen (of mengsels daarvan) toxisch zijn. De nieuwe methoden bieden de kans in kaart te brengen in welke biologische systemen de belangrijkste veranderingen optreden na blootstelling aan gif. Processen die verstoord worden, maar ook beschermende mechanismen kunnen zo geduid worden. Naast deze inzichten kan tegelijkertijd ook de normale gang van zaken in de cel op moleculair niveau bestudeerd worden.

Na blootstelling aan een mengsel van giftige stoffen kunnen er effecten optreden die niet te verwachten waren op basis van de effecten van de afzonderlijke stoffen, zgn. interacties. Mengsels kunnen dus leiden tot onverwachte schadelijke effecten. Het vaststellen van interacties na blootstelling aan combinaties van stoffen vergt op dit moment zeer uitgebreide experimenten. Dit komt met name omdat er slechts zelden een goed begrip is van de werkingsmechanismen van de individuele componenten, laat staan van mogelijke beïnvloeding van stoffen van elkaar. Toxicogenomics kan helpen de werkingsmechanismen op te helderen. Bovendien biedt het meten van duizenden parameters meer kans op het vinden van effecten die de verschillend behandelde groepen onderscheiden dan het kleine aantal huidig beschikbare parameters.

Doelen van dit onderzoek

Van alle eerder beschreven mogelijke toepassingen is in dit proefschrift met name beschouwd of toxicogenomics kan bijdragen aan:

a) het onderzoeken van mechanismen van toxiciteit

b) een vroege herkenning van (toxische) effecten bij een blootstelling aan lage dosering, en de identificatie van nieuwe markers hiervoor

c) het aantonen en verklaren van effecten van mengsels van toxische stoffen

d) succesvolle toepassing van *in vitro* modellen en reductie van proefdiergebruik

Daarnaast zijn technische aspecten van de toxicogenomics zoals reproduceerbaarheid en gevoeligheid bepaald. De relatie tussen de nieuwe technologieën onderling en de gangbare methoden is onderzocht.

Opbouw van het proefschrift en resultaten

Hoofdstuk I bevat de Engelstalige introductie, die een uitgebreide beschrijving geeft van de mogelijke toepassingen van toxicogenomics methoden. Daarnaast zijn de methoden in wat meer detail besproken. De feitelijke studies zijn beschreven in de **hoofdstukken II tot en met VII,** waarna in **hoofdstuk VIII** de resultaten bediscussiëerd worden.

Allereerst (**hoofdstuk II**) is bepaald of gen- en eiwitexpressie veranderingen na blootstelling aan de levertoxische stof broombenzeen (BB) aansluiten bij de bestaande inzichten. Verder is onderzocht welke nieuwe inzichten de technieken konden verschaffen met betrekking tot de moleculaire mechanismen van het ontstaan van celdood in de lever.
Broombenzeen is een industrieel toegepaste organisch chemisch middel, en werd in deze studie gebruikt omdat het een stof is met welbekende levertoxische werking. Ratten werden eenmalig gedoseerd met BB, en na 24 uur werd de genexpressie bepaald voor meer dan 3000 verschillende genen in de lever, gebruikmakend van DNA microarray's. Daarnaast zijn eiwitten in diezelfde levers geanalyseerd met geavanceerde proteomics technologieën.
Onze studie bevestigde dat glutathion (GSH) in de lever een centrale rol speelt in het ontgiften van broombenzeen en bescherming tegen schade. Ook werden aanwijzingen gevonden voor de verstoring van processen als de aanmaak van cholesterol en vetzuren. Deze processen waren nog niet eerder gerelateerd aan deze vorm van levertoxiciteit. Diverse bewijzen werden gevonden voor een verhoogde aanmaak en afbraak van eiwitten, hetgeen duidelijk naar voren kwam met de proteomics technieken.

Als vervolg op de eerste studie bepaalt de studie in **hoofdstuk III** of de veranderingen in genexpressie na blootstelling aan BB afhankelijk zijn van de dosering. Voorts is bepaald hoe de veranderingen optreden in de tijd, 6, 24 en 48 uur na een orale dosering. De DNA microarray's bleken in staat veranderingen aan te tonen bij 2,5 tot 5-voudig lagere doseringen dan de conventionele technieken. Enkele veranderingen, zoals expressie van heem oxygenase, werden al 6 uur na dosering gemeten.

In **hoofdstuk IV** werd gebruik gemaakt van de genexpressie metingen uit de voorgaande studie met daaraan toegevoegd metabolomics analyses. Hiervoor werden bloed plasma en urine van dezelfde ratten geanalyseerd met NMR spectroscopie. Op een gevoelige manier zijn zo honderden metabolieten in deze monsters gemeten. De monsters uit de verschillende behandelgroepen waren te onderscheiden met wiskundige patroonherkenningstechnieken. Deze studie toonde aan dat een geïntegreerde aanpak mogelijk maakte om de moleculaire profielen van individuele dieren met een verschillende graad van celdood in de lever van elkaar te onderscheiden. Zo werden moleculen geïdentificeerd die typerend zouden kunnen zijn voor celdood in de lever (toxiciteitsmarkers). De betrokkenheid van enkele biochemische processen, zoals aminozuur metabolisme en glycolyse werd op moleculair niveau gerelateerd aan deze vorm van levertoxiciteit.

Na de bestudering van effecten van broombenzeen, een industrieel toegepaste product en modelstof voor levertoxiciteit, werden de effecten van twee veelvuldig gebruikte chemicaliën onderzocht op genexpressie niveau in de lever. Een van de doelen hiervan was te bepalen hoe specifiek de genexpressie veranderingen eigenlijk waren voor stoffen die schade in de lever veroorzaakten.

Hoofdstuk V beschrijft welke veranderingen er optraden nadat ratten gedurende 28 dagen blootgesteld werden aan benzeen. Benzeen wordt industrieel toegepast bij de productie van o.a. verf, inkt, en rubber, en komt voor in uitlaatgassen en sigarettenrook. Het is bekend dat de kritische effecten van benzeen gevonden worden in het bloed en beenmerg. Benzeen is carcinogeen en kan de kans op leukemie verhogen. Met de conventionele toxicologie werd bevestigd dat benzeen bloedvergiftiging veroorzaakt, gezien de reductie van het aantal witte bloedcellen en van het gewicht van de thymus en milt. In de lever daarentegen werden, behalve een kleine gewichtstoename, geen microscopische veranderingen geconstateerd. Desalniettemin werd met DNA microarray's gevonden dat de genexpressie in de lever veranderde, zelfs bij een lage dosering. Zo kon inzicht verkregen worden in welke processen betrokken zijn bij de reactie op blootstelling aan benzeen. Met name enzymen betrokken bij de omzetting van lichaamsvreemde stoffen (zoals benzeen zelf) werden geactiveerd. Meer onderzoek moet aantonen of dit de schadelijke effecten van benzeen verhoogt of juist verlaagt. Het profiel van genexpressie bleek gevoeliger effecten aan te tonen dan de gangbare methoden. Ook de profielen van metabolieten in de urine waren gevoelig te onderscheiden op basis van de blootstelling.

Hoofdstuk VI beschrijft een onderzoek als in hoofdstuk V, waarbij nu de toxische effecten van trichloorethyleen (TCE) onderzocht werden. TCE is een industrieel veel toegepast chemisch product, ontvetter, oplosmiddel, maar ook toegepast in correctievloeistof (bijv. typ-ex). Het kritische effect van TCE wordt gevonden in de nieren. Verder kan het ook schade in de lever veroorzaken. De dosisafhankelijke veranderingen in genexpressie die gevonden werden in de lever na blootstelling aan TCE gedurende 28 dagen, hebben gevolgen voor diverse processen in de lever, maar ook elders in het lichaam. Zowel enzymen die TCE activeren tot giftige metabolieten, als ook ontgiftende enzymen werden geactiveerd.

Door benzeen en TCE veroorzaakte veranderingen in de expressie van levergenen werden vergeleken met effecten van BB en van een hoge dosering paracetamol (acetaminophen), beschreven door Heinloth *et al.*, (2004). BB en paracetamol staan bekend als stoffen die specifieke schade (acute celdood) in de lever veroorzaken. De genexpressie veranderingen door benzeen en TCE overlapten gedeeltelijk met de effecten van BB en paracetamol. Echter, BB en paracetamol veroorzaakten meer en duidelijkere veranderingen in genexpressie. Voor alle onderzochte stoffen kon een karakteristiek patroon van veranderingen verkregen werden, hetgeen de bruikbaarheid van de toxicogenomics methoden bevestigt.

In **hoofdstuk VII** is voor het eerst met DNA microarrays onderzoek gedaan naar effecten van mengsels van giftige stoffen. Ratten werden blootgesteld aan benzeen, trichloorethyleen of een combinatie daarvan in diverse doseringen, en de levers werden geanalyseerd met de DNA microarray's. De conventionele toxicologische bepalingen bleken slechts weinig effecten aan te tonen in de lever. De DNA microarray's daarentegen bewezen dat er veel genexpressie veranderingen optraden na blootstelling. De genen die het meest karakteristiek waren voor de effecten van benzeen en TCE uit de vorige studies werden opnieuw opgemerkt. Bestudering van de effecten van mengsels van benzeen en TCE gaf hoofdzakelijk aan dat genexpressie effecten van trichloorethyleen versterkt werden door toevoegen van benzeen.

Conclusies

Uit dit proefschrift blijkt dat toxicogenomics mogelijkheden biedt om mechanismen van (lever)toxiciteit te verhelderen. Duidelijk is geworden dat toxicogenomics op dit moment met name geschikt is om hypothesen te genereren rondom werkingsmechanismen van toxiciteit. Deze dienen dan met aanvullend onderzoek gestaafd te worden (*in vivo* en *in vitro*). Er werden voorspelbare, maar ook onverwachte effecten van welbekende gifstoffen zoals broombenzeen, benzeen en trichloorethyleen gevonden. Een voorbeeld daarvan vormt de beïnvloeding van het vetzuur- en cholesterol metabolisme door de onderzochte chemicaliën.

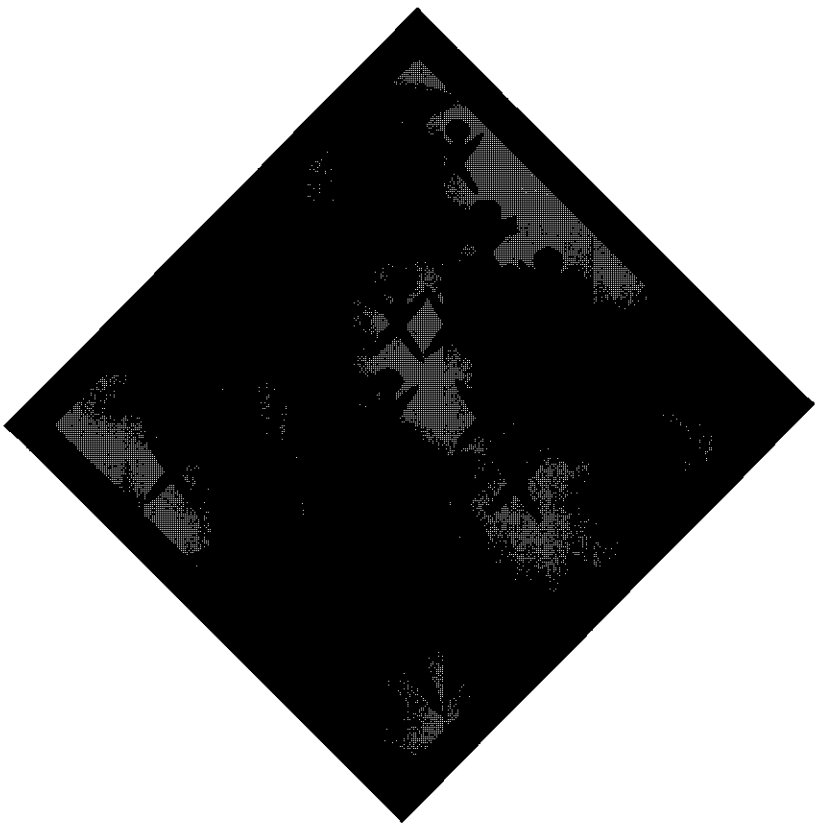
Dit proefschrift toont aan dat het is mogelijk om gevoeliger dan met de gangbare methoden in een vroeg stadium effecten te identificeren. Zo kan bij meer realistische, lage doseringen inzicht verkregen worden in de processen die optreden na blootstelling aan toxische stoffen.

Het tweede deel van dit proefschrift laat zien hoe toxicogenomics gebruikt kan worden om de toxiciteit van mengsels van stoffen te onderzoeken. Met name door het verhelderen van werkingsmechanismen kunnen onderbouwde hypotheses over wederzijdse beïnvloeding van stoffen gevormd worden.

De studies laten een voorbode zien van hoe toxicogenomics het gebruik van proefdieren zou kunnen reduceren en verfijnen. Kennis en inzicht in de werking van giftige stoffen kunnen leiden tot vervanging van standaard experimenten door verfijnde en toegesneden proeven. Toxicogenomics kan mogelijk een alternatief voor proefdiergebruik bieden wanneer het toegepast wordt in combinatie met (*in vitro*) modellen. Op de wat langere termijn zijn aldus verkregen inzichten misschien voldoende om de volksgezondheid te waarborgen.

De integratie van de nieuwe genomics technologieën zou diepgaande inzichten kunnen bieden in processen in de cel en verstoringen hiervan na blootstelling aan toxische stoffen. De uitdaging voor de toekomst is om inzicht te krijgen in alle stappen van de ingewikkelde werkingsmechanismen van toxiciteit. Door de schakelingen, communicatie en logistieke systemen te bestuderen en te beschrijven zou uiteindelijk een model opgesteld kunnen worden van het fijne raderwerk in een cel. Zo'n model zou dan gebruikt kunnen worden om te voorspellen wat de effecten zijn van invloeden van buitenaf, waaronder ook gifstoffen.

Dankwoord



Dankwoord

In het begin van dit millennium, in januari 2000, was dit nog slechts een leeg schrift. Blanco, zonder introductie, inhoud of zelfs maar een titel. Dankzij de inzet van velen kon ik dit schrift vullen tot een proefschrift. Ik wil iedereen bedanken die daaraan een bijdrage heeft geleverd!

Enkele mensen verdienen een bijzondere vermelding! Allereerst wil ik mijn ouders bedanken dat ze mij als een nieuwsgierig en eigenwijs mannetje op de wereld hebben gezet! Samen met mijn broer en zussen werd ik altijd geholpen om groter en (eigen)wijzer te worden, ook nadat ik niet meer te houden was en uitvloog naar de universiteit.

Eenmaal bij TNO Voeding was Ben van Ommen de grote gangmaker. Ben bood me een interessante baan aan en omdat toxicologie toch een soort 'gestoorde biologie' is, leek het mij als moleculair bioloog wel wat. Met baanbrekende technieken zouden we wel 'even' de expressie van enkele duizenden genen onderzoeken na blootstelling aan giftige stoffen. Massa's publicaties zouden volgen, dus kon ik het beste meteen ook maar promoveren! Nou Ben, het boek is gevuld! Het was niet altijd zo simpel, maar gelukkig blies je met je eeuwige optimisme alle vuiltjes uit de motor en hield de vaart erin! Ben, bedankt voor je enthousiasme en inspiratie!

De eerste kennismaking met promotor Peter van Bladeren maakte me snel duidelijk dat hij naast mijn project nog wel wat meer zaken behartigde. Een mooie kamer, eigen secretaresse, sigaren en weinig tijd, hij leek de directeur van TNO wel! Ondanks al die andere zaken heb ik altijd tijd en belangstelling gekregen. Peter, bedankt dat je kritisch de juiste richting bewaakte en ook vanuit Zwitserland betrokken bleef.

De betrokkenheid van John Groten, mijn afdelingshoofd bij TNO, werd nog groter door zijn aanstelling als hoogleraar in Wageningen. Vooral het tweede gedeelte van dit proefschrift en de combinatietoxicologie kregen volop aandacht. John, bedankt voor je inspanning om kritisch en nauwgezet de artikelen te helpen verfijnen. Leuk dat je bij mij voor het eerst als promotor optreedt.

Het werk begon ruim vier jaar geleden met het vermenigvuldigen van duizenden rattengenen, en het glaasjes 'spotten' in het lab van Frank Schuren, samen met Annemiek, Evelyn en Ted. Er werden talloze technische euvels opgespoord en opgelost en de DNA microarray analyse ging echt werken. Het vertrek van de vrolijke pioniers van de array's bij TNO was steeds een aderlating. De hulp van o.a. Mieke en Alie maakte veel goed. Alle betrokken AMGT collega's hartelijk bedankt!

Met name ons labhoofd Michèle en ook Anja, Hester en Dionne verrichtten veel hak- en stampwerk en isoleerden RNA voor op de microarrays. Gelukkig bleken toxicogenomics proeven met relatief weinig monsters erg veel antwoorden te kunnen leveren. Hierdoor bleef het aantal ratten die in dit onderzoek een nog onmisbare rol vervulden, beperkt! Ik ben dan ook blij dat we veel monsters konden verzamelen uit al lopende studies. De grote hoeveelheid data bleek ook de grote uitdaging, namelijk om uit die miljoenen getalletjes zinnige informatie te halen. Het overgrote deel van mijn werk bestond dan ook uit data-analyse in de computer. Dit was soms aanleiding voor collega's om te twijfelen of ik wel een échte AIO was, en of ik eigenlijk wel een labjas had! De ervaringsdeskundigen uit onze genomics groep konden hierover goed meepraten, maar gelukkig ook over vele andere onderwerpen. Bedankt allemaal voor een vrolijke werkomgeving.

Rob, bedankt voor het samen schrijven van artikelen, het delen van frustraties en de kritische beschouwingen tijdens het rondje! Het was ook leerzaam van dichtbij te zien hoe een nèt iets oudere en wijzere onderzoeker de wervelwind van projecten ingezogen werd. Fijn dat je mij als paranimf wilt bijstaan. Dat geldt ook voor Marjan. Het was geweldig om samen alles van het begin af mee te maken. Dat jij me op deze baan ooit attendeerde is ook niet geheel onbelangrijk. En omdat je wat eerder promoveerde kon ik mooi parasiteren op jouw en Miriam's ervaring in het produceren van een proefschrift. Je was altijd de bron van nuttige en betrouwbare informatie, toegang tot de vakgroep toxicologie in Wageningen en vele artikelen!.
Marinus, enthousiaste whizz-kid, het is aan jou te danken dat ik me nu een oude, ingedutte en chagrijnige vent voel. Maar toch bedankt voor je energieke inzet en (on)gevraagde wetenswaardigheden. Robert-jan, bedankt voor jouw analyses voor onze gezamenlijke publicaties (te vernieuwend voor menig reviewer?) en veel succes met het afronden van je metabolomics proefschrift.

De stagiaires wil ik bedanken voor hulp en appeltaarten, en met name Marieke Bart voor vele microarray hybridisaties. Diana Jonker, Andreas Freidig en Heleen Wortelboer, Joop van Nesselrooij en Monique Slijper (Utrecht) bedank ik hartelijk voor hun bijdragen aan publicaties. Talloze (oud) collega's hebben me op diverse manieren geholpen met het onderzoek, en met name noem ik Ana, Cyrille, Wilfred, Johan, Yvonne, Maria, Nicole, Han, Jan, Birol, Dimitri, Miriam, Rianne, Florence, Freek, Bert, Sonia, Marie-Jose, Nico, Truus, Paula, Richard, Michiel, Almira, Azz-Eddine, Lisette, Pol, Cor, Marco, Sabina, Bianca, Renger, Florian, Eric, Anne-Marie, Marloes, Jolanda, Karin en die ene collega wiens naam ik nu even ben vergeten! Het was fijn en nuttig ook andere TNO promovendi te kennen, waaronder Martijn, Micha, Kitty, Bart, Barry, Wendy, Joline. En in onze groep de aanwinsten Anne en Ashwin, de hoop op diepgaand onderzoek is nu op jullie gevestigd! I would also like to thank dr. Angela Slitt and dr. Curtis Klaassen from Kansas City for our valuable collaboration.

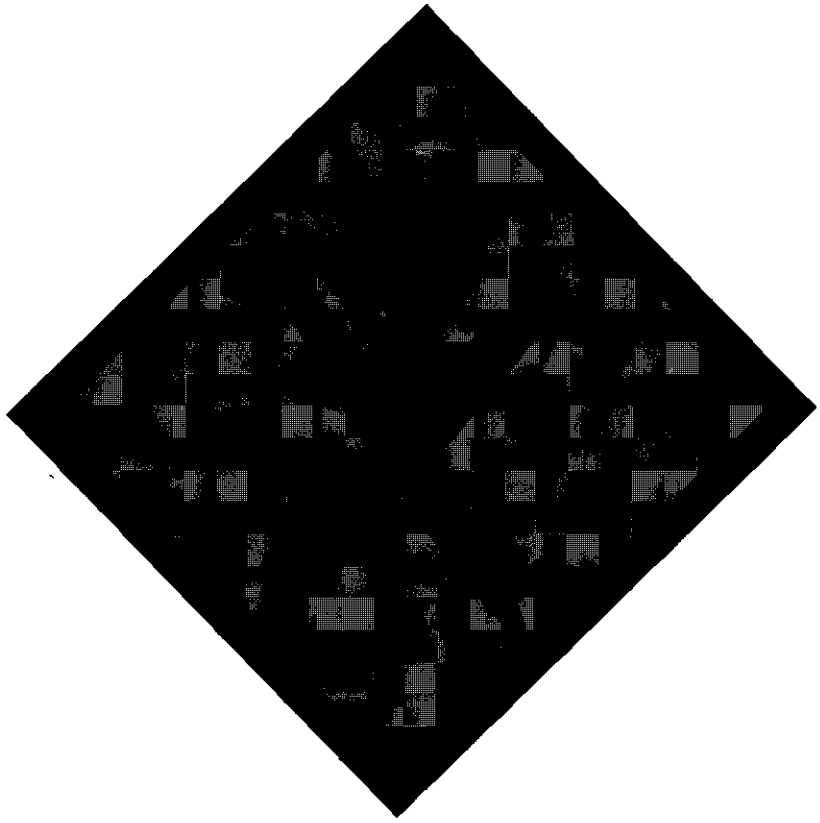
Goed werk kan niet geleverd worden zonder ontspanning en plezier buiten werktijd. Omdat ik de afgelopen jaren zoveel moois heb meegemaakt, kan ik het belang van promoveren wel enigszins relativeren. Ik wil natuurlijk mijn ouders, Saskia, Maarten en Veerle en ook Hélène en Han, Olivia, de oma's en andere familie hartelijk bedanken voor al hun aandacht.

Gisela, jou kan ik eigenlijk op papier niet genoeg bedanken. Vooral het laatste jaar eiste dit proefschrift veel van onze aandacht en toch al schaarse tijd op. Gelukkig kon je me helpen om realistische doelen en prioriteiten te stellen. En, jouw 'g.a.a.p.-index' zou wel eens een nieuwe standaard kunnen worden bij het toetsen van de toegankelijkheid van artikelen. Onder de Nederlandse samenvatting zou ook jouw naam moeten staan. Je vakantie en veel vrije tijd werden dit jaar opgeofferd aan mijn werk en zonder jou had dit project vast veel langer geduurd!

Tenslotte ons meisje, jij kwam vast ook wel eens aandacht tekort. Vanaf het eerste uur wist je echter zeer goed duidelijk te maken dat er méér is dan toxicogenomics! Ik hoop dat jij, en elk kind op deze wereld, net als ik de kans krijgt om te onderzoeken en ontdekken hoe het leven in elkaar zit, stap - voor - stap.....

Wilbert

About the author



About the author

Wilbert H.M. Heijne werd geboren op 21 mei 1976 te Genève (CH). Na verhuizing in 1984 volgde hij basisonderwijs en middelbaar onderwijs in Nederland, en haalde in 1994 het Gymnasium diploma aan de Jansenius Scholengemeenschap te Hulst.
Aansluitend studeerde Wilbert moleculaire wetenschappen aan de universiteit van Wageningen (biotechnologische specialisatie) met als afstudeeronderwerpen moleculaire biologie, virologie, genetica, biochemie en bioinformatica. In 1999 werkte hij 6 maanden bij dr. M.Saier aan de universiteit van San Diego (UCSD), U.S.A., aan de analyse van transporteiwitten met moleculaire biologie en bioinformatica. Zijn werk tijdens deze onderzoeken droeg bij aan enkele wetenschappelijke publicaties.
Na het behalen van de bul in 1999, kreeg Wilbert een aanstelling bij TNO Voeding in Zeist. Hier werkte hij van januari 2000 tot september 2004 aan toxicogenomics studies, en voerde het onderzoek uit dat beschreven is in dit proefschrift. Allereerst werd een onderzoeksvoorstel opgesteld en vervolgens werden studies uitgevoerd om de nieuwe discipline Toxicogenomics te verkennen. Vanaf het eerste uur droeg Wilbert bij aan de ontwikkeling van de DNA microarray technologie bij TNO. De toepassing van deze nieuwe technologie in de toxicologie resulteerde in wetenschappelijke publicaties, overzichtsartikelen en presentaties op congressen als van de Amerikaanse Society of Toxicology (SOT). Wilbert participeerde in een aantal workshops over bioinformatica, data analyse en biostatistiek, en kreeg in 2003 een SOT reisbeurs en de prijs voor de beste presentatie op het jaarcongres van de Nederlandse Vereniging voor Toxicologie.

Wilbert H.M. Heijne was born on May 21st in Genève (Switzerland). He received primary and secondary education (gymnasium) in the Netherlands and graduated in 1994 at the"Jansenius" Secondary school in Hulst. He studied Molecular Sciences at Wageningen University (NL), (specialisation biotechnology), with majors in molecular biology, virology, genetics, biochemistry and bioinformatics. In 1999, he worked for 6 months at the university of San Diego (UCSD), U.S.A. in the lab of dr. M.Saier, to analyse transport proteins with molecular biology and bioinformatics, resulting in several scientifical publications.
After graduation in 1999, Wilbert joined the TNO Nutrition and Food Research institute in Zeist (NL). As a research fellow, he participate in toxicogenomics studies and performed the work described in this thesis. From the start at TNO, Wilbert was involved in the development of the DNA microarray technology. The applications of these methods in toxicology were described in peer-reviewed scientific publications, reviews and oral presentations at conferences like the annual meeting of the U.S. Society of Toxicology (SOT). Wilbert participated in various workshops about bioinformatics, data analysis and biostatistics, and in 2003, he received a SOT travel award and the award for the best presentation at the annual meeting of the Dutch Society of Toxicology (NVT).

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To be submitted

Heijne WHM, Stierum RH, Leeman, WR and van Ommen B (2004)
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- EMS 2001, Seattle, U.S.A.

Glossary

DNA Microarray: Tool to measure levels of gene expresssion (mRNA molecules); glass microscope slide with thousands of (arrayed) spots containing tiny amounts of DNA fragments. Each spot contain DNA fragments corresponding to a different gene.

Functional genomics technologies: technologies that study the functions of the genome in relation to its structure and composition. These include genomics, transcriptomics, proteomics and metabolomics.

Gene Functional part of the DNA (chromosomes) that retains the code for the synthesis of a protein

Hepatotoxicity: Liver toxicity

Hematology: analysis of blood

Hematotoxicity: blood toxicity

Metabolomics Studying levels of metabolites (small molecules) under different circumstances in biological samples such body fluids or tissue samples

Messenger RNA (mRNA): molecule that transfers information from the DNA (gene) to the location in the cell where the proteins are assembled using this information

Nephrotoxicity: kidney toxicity

Proteomics: Studying protein contents in biological samples such as tissue or body fluids

Toxicogenomics: Integration of functional genomics technologies in toxicology

Toxicology: Studying harmful effects of poisonous substances in the (human) body

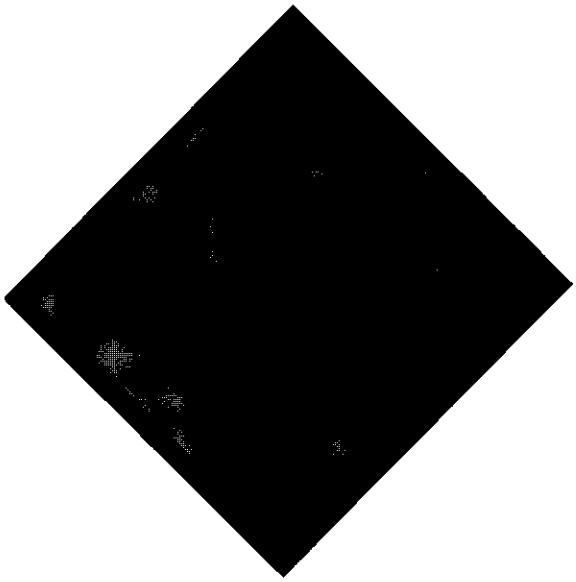
Transcriptomics: Studying transcription (activity) of genes under different circumstances in biological tissue samples. This is achieved by measuring mRNA levels

Abbreviations

Abbreviation	Description
AGT	alanine-glyoxylate aminotransferase
ALAT	alanine aminotransferase
ALP	alkaline phosphatase
APAP	acetaminophen (paracetamol)
APR	acute phase response
ASAT	aspartate aminotransferase
ATSDR	Agency for Toxic Substances and Disease Registry
B	benzene
BB	bromobenzene
bDNA	branched DNA signal amplification (assay)
cDNA	complementary (copy) DNA
CDNB	1-chloro-2,4-dinitrobenzene
CGH	comparative genome hybridization
CO	corn oil (vehicle control)
CYP	cytochrome P450 enzyme
ELISA	enzyme-linked immunosorbent assay
FDA	U.S. Food and Drug Administration
FISH	fluorescent in situ hybridisation
GC	gas chromatography
GSH	reduced glutathione
GSH	glutathione
GSSG	oxidised glutathione
GST	glutathione S-transferase
i.p.	intraperitoneal
ICAT	Isotope-coded affinity tagging
LDH	lactate dehydrogenase
MALDI-TOF	Matrix Assisted Laser Desorption Ionisation - Time Of Flight
mRNA	messenger RNA
MS	mass spectrometry
n.s.	not statistically significant
NMR	Nuclear magnetic resonance spectroscopy
OECD	Organisation for Economic Co-operation and Development
PC	principal component
PCA	principal component analysis
PCDA	principal component discriminant analysis
ROS	reactive oxygen species
RT-PCR	real-time reverse transcriptase polymerase chain reaction
SAGE	serial analysis of gene expression
SELDI	Surfance-enhanced laser desorption/ionization
SNP	single nucleotide polymorphism
TCA	trichloroacetic acid
TCE (T)	trichloroethylene
TNO	Netherlands Organisation for Applied Scientific Research
UT	untreated

Genes

Symbol	Gene name
Abcc	ATB binding cassette containing transporter
Acat1	acyl-CoA acetyltransferase
Actb	Actin beta
Adh	Alcohol dehydrogenase (Adh1,....)
Afar	Aflatoxin aldehyde reductase
Ahr	Aryl hydrocarbon receptor
Alas2	aminolevulinic acid synthase 2
Aldh	Aldehyde dehydrogenase (Aldh1, ...)
Apo	Apolipoprotein (ApoB, ApoE)
Atpase	ATP synthase
Bhmt	Betaine homocysteine methyltransferase
Cd36	Cd36 antigen (enzyme)
Cdh	Cadherin
Cps	carbamoyl-phosphate synthase
Cyp	Cytochrome P450 enzyme (Cyp2a1, Cyp2e1, Cyp2c,...)
Dci	dodecenoyl CoA isomerase
Dopa	D-dopachrome tautomerase
Ephx1	epoxide hydrolase (microsomal)
Feh, Fel	ferritin heavy or light chain
G6pc	glucose-6-phosphatase
G6pd	glucose-6-phosphate dehydrogenase
gadd45	Growth arrest and DNA damage protein 45
Gapdh	Glyceraldehyde 6-phosphate dehydrogenase
Gcl c	gamma cysteine ligase (gamma-glutamylcysteine synthase) light chain
Gst	glutathione-S-transferase (Gsta, Gstm,...)
Hao	Hydroxy acid oxidase
Hnf	hepatic (hepatocyte) nuclear factor (Hnf3, Hnf4,Hnf6)
Ho-1	heme oxygenase
Hpd	hydroxyphenylpyruvate dioxygenase
Hsp	Heat shock protein (Hsp60, Hsp70,..)
Lcat	Lecithin-cholesterol acyltransferase
L-Fabp	liver fatty acid binding protein
Lisch7	liver-specific transcription factor
Me1	Malic enzyme1
Mpo	myeloperoxidase
Mrp	Multi-drug resistant protein (Mrp1, Mrp2, Mrp3,...)
Mt	Metallothionein (Mt1, Mt2, Mt3,...)
Myd116	Myeloid differentiation primary response gene 116
Nqo1	NAD(P)H dehydrogenase, quinone 1
Oat	Ornithine aminotransferase
PPAR	Peroxisome-proliferation activated receptor (alpha, gamma,..)
RXR	Retinoid - X- receptor
Slc	Solute carrier protein
Tat	Tyrosine aminotransferase
Timp1	Tissue inhibitor of metalloproteinase
Ugt	UDP-glucuronosyltransferases



Omslagillustratie
Dit proefschrift illustreert hoe aan de hand van metingen van activiteit van genen de giftigheid van stoffen onderzocht kan worden. Het toont ook dat er nog een hele weg te gaan is tot alle vragen opgelost zijn, stap voor stap!

Cover illustration
This thesis demonstrates how the toxicity of substances can be determined with the aid of gene expression measurements. It also shows that we are a long way from solving all the questions, step-by-step.

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