



National Institute for Public Health  
and the Environment  
*Ministry of Health, Welfare and Sport*

## **Toxicogenomics in carcinogenicity hazard assessment**

Report 340700004/2010

L.G. Hernández | W.C. Mennes | J. van Benthem |  
M. Luijten



National Institute for Public Health  
and the Environment  
*Ministry of Health, Welfare and Sport*

## **Toxicogenomics in carcinogenicity hazard assessment**

RIVM Report 340700004/2010

## Colophon

© RIVM 2010

Parts of this publication may be reproduced, provided acknowledgement is given to the 'National Institute for Public Health and the Environment', along with the title and year of publication.

L.G.Hernández  
W.C.Mennes  
J. van Benthem  
Dr.M.Luijten

Contact:  
M.Luijten  
Laboratory for Health Protection Research  
[mirjam.luijten@rivm.nl](mailto:mirjam.luijten@rivm.nl)

This investigation has been performed by order and for the account of Ministry of Health, within the framework of project V/340700 Advisering en kennisbasis CMR

## Abstract

### **Toxicogenomics in carcinogenicity hazard assessment**

Research performed by the RIVM demonstrates that toxicogenomics data can help improve the assessment of the carcinogenic potential of chemicals. Toxicogenomics is a field of science in which microarray technology is used to study the response of cells to toxic substances through the analysis of expression profiles at the level of mRNA, proteins or metabolites. The primary aims of such research are to elucidate molecular mechanisms that result in the induction of a toxicological effect and to identify molecular biomarkers that predict toxicological hazard.

In most countries, the use of specific chemicals is not allowed unless it can be shown that the substance does not pose any risk to both human health and the environment. Test strategies for toxicity testing consist of *in vitro* assays and animal studies. Toxicity data obtained for a specific chemical are used to assess its potential impact on human health. The assessment of the toxic potential of a chemical is a complicated, time-consuming and animal-demanding process.

Implementation of toxicogenomics in current testing strategy for carcinogenicity may have a significant impact. Toxicogenomics data can be used to define mechanisms of action and improve interspecies extrapolations. In addition, it contributes to alternatives to animal testing by reducing and refining animal use in carcinogenicity studies.

**Key words:**

toxicogenomics, risk assessment, genotoxicity, carcinogenicity

## Rapport in het kort

### **Toxicogenomics en het herkennen van kankerverwekkende stoffen**

Volgens de wet moet voor elke chemische stof worden aangetoond dat deze geen risico vormt voor de gezondheid van de mens en voor het milieu. Dit gebeurt door een stof te testen op schadelijke effecten (toxiciteit), zowel in gekweekte cellen (*in vitro*) als in proefdieren (*in vivo*). De verkregen gegevens worden vervolgens omgerekend om een inschatting van de toxiciteit voor de mens te maken. Dit proces is ingewikkeld en tijdrovend, en kost bovendien veel proefdieren.

Voorliggend rapport laat zien dat toxicogenomics kan helpen om vast te stellen of een chemische stof kankerverwekkend is voor de mens. Blootstelling aan een schadelijke stof leidt tot veranderingen in de genactiviteit die met toxicogenomics kunnen worden gemeten.

Het RIVM heeft geconstateerd dat toxicogenomics een rol kan spelen bij het herkennen van kankerverwekkende stoffen via het ontrafelen van werkingsmechanismen. Hierdoor wordt de risicobeoordeling beter onderbouwd. De vertaalslag van proefdier naar mens wordt eenvoudiger en het proefdiergebruik wordt teruggedrongen omdat minder dieren nodig zijn en het ongerief van de proefdieren wordt verminderd.

Trefwoorden:

toxicogenomics, risicobeoordeling, genotoxiciteit, carcinogeniteit

## Contents

Summary—6

**1 Introduction—7**

**2 Carcinogenicity testing—8**

2.1 Genotoxicity testing strategy—8

2.2 Cancer bioassay—8

**3 Toxicogenomics—10**

3.1 Mechanism of action—10

3.2 Predicting toxicity—10

**4 Toxicogenomics in carcinogenicity hazard assessment—13**

4.1 Toxicogenomics in genotoxicity testing—13

4.1.1 Possible applications of toxicogenomics in in vitro genotoxicity tests—13

4.1.2 Possible applications of toxicogenomics in in vivo genotoxicity tests—13

4.1.3 Example of toxicogenomics in genotoxicity hazard assessment—14

4.2 Cancer bioassay—15

**5 Toxicogenomics from a regulatory perspective—17**

5.1 Requirements for implementation of toxicogenomics in risk assessment strategies—17

5.2 What should be done to satisfy regulators and successfully implement toxicogenomics in risk assessment?—19

5.2.1 Standardization—19

5.2.2 Quantitative aspects—19

**6 Conclusions—21**

**References—22**

## Summary

Cancer is one of the leading causes of death in the Western world. Many chemicals have the potential to cause cancer. Given the increasing number of new chemicals that are synthesized each year, it is important to test chemicals for their carcinogenic potential before they can be marketed. For this, the lifetime bioassay using rats and mice is routinely used. However, over the years this assay has clearly come to reveal several drawbacks: large numbers of animals are used, and the assays are slow, insensitive and expensive. On top of this, there is considerable scientific doubt about the reliability of the assay, since too many false positive results (so-called rodent carcinogens) have been observed. Therefore, alternative methods to predict carcinogenic features of compounds are in demand.

In this regard, new technologies such as toxicogenomics could help improve the current test strategy. Toxicogenomics combines toxicology with high-throughput molecular profiling technologies such as transcriptomics, proteomics and metabolomics. Toxicogenomics aims to elucidate molecular mechanisms that result in the induction of a toxicological effect, and to identify molecular biomarkers that predict toxicity. In this report, we explored the applicability of toxicogenomic-based screens in risk assessment and investigated whether improvements to the current test strategy are possible.

In genotoxicity testing, the added value of toxicogenomics is limited. However, in carcinogenicity testing toxicogenomics may have a significant impact. It could be used to define mechanisms of action and improve interspecies extrapolations by bettering our understanding of the relevance of various animal dose-response data to actual human exposures. It can also be utilized in a quantitative manner to derive potency estimates. In addition, implementation of toxicogenomics contributes to alternatives to animal testing by reducing the number and discomfort of animals used in carcinogenicity studies.

To date, toxicogenomics is predominantly used as a tool for the elucidation of a mechanism of action and thus for hazard identification of a chemical. In order to integrate toxicogenomic endpoints in quantitative risk assessment it is essential to know whether relevant changes in gene expression occur in a dose-dependent manner and how these changes should be interpreted in terms of adversity. For this, more dose-response and time-course studies in large sets of compounds encompassing multiple toxicological endpoints are needed to fill the existing data gaps. In addition, (worldwide) standardization of several aspects of toxicogenomics is needed in order to be utilized in risk assessment.

## 1 Introduction

Cancer is one of the leading causes of death in developed countries with an estimated 7 million deaths from cancer per year (IARC 2008). The exposure to chemicals may be an important contributor to this high incidence of cancer and with the constant development of new chemicals, the assessment of the carcinogenic potential of compounds is of extreme importance. This assessment is done by the evaluation of the cancer risk for humans which has a qualitative (hazard identification) and a quantitative (dose-response analysis) component. Genotoxicity assays are currently used for the hazard identification of large numbers of chemicals for possible carcinogenic properties. Normally, genotoxicity tests are applied according to a (tiered) test strategy whereby a positive result triggers further testing and an *in vitro* positive result needs *in vivo* confirmation. A substance is suspected to be carcinogenic based on the results of *in vitro* and *in vivo* genotoxicity tests and a cancer bioassay is performed to obtain hazard confirmation and information with regards to cancer potency (*i.e.* dose-response analysis). The cancer bioassay involves a two-year exposure to a chemical using 50 animals per dose per sex with a minimal of 3 doses, in addition to the vehicle control for two species. In general, dose-response assessment involves an evaluation within the range of tumour observations to identify the toxicological effects responsible for carcinogenesis by analyzing and evaluating the risk estimates across all tumour types, reviewing any available information on the mode of action, and evaluating the anticipated human relevance associated with each tumour type. Once the most sensitive tumour endpoint is selected, it is used for a dose-response assessment which is the extrapolation to lower dose levels that mimic human exposures (EC 2003).

The current test strategy is not without disadvantages, given that genotoxicity tests are not perfect in predicting the carcinogenic potential of chemicals and are not designed to detect non-genotoxic carcinogens. As such, some compounds are misclassified. In addition, the assessment of carcinogenicity is a complicated, time-consuming, and animal-demanding process. In this regard, new technologies such as toxicogenomics could help improve the current test strategy. Toxicogenomics is the application of microarray technology to toxicology with the aim to identify molecular mechanisms that result in the induction of a toxicological effect, and to derive molecular expression patterns (*i.e.* molecular biomarkers) that predict toxicity. In this report, we will explore the applicability of toxicogenomics in risk assessment and explore whether improvements to the current test strategy are possible.

## 2 Carcinogenicity testing

Carcinogenesis is a multi-step process where tumours grow through a process of clonal expansion, within which various mutations accumulate and eventually enable a cell to become malignant, *i.e.* to have the ability to invade neighbouring tissues and metastasize (Vogelstein and Kinzler 1993). For this reason, carcinogenesis can be divided into three stages: initiation, promotion and progression (Vogelstein and Kinzler 1993). Initiation involves the induction of gene and/or chromosomal mutations. Promotion involves the stimulation of clonal expansion where an increase in cell proliferation gives rise to daughter cells that contain the initiated damage, in addition to any other accumulated genetic mutations. Finally, progression involves the stepwise transformation of a benign tumour to malignancy. In this last step, the cells have often karyotypic changes and an increase in growth rate, invasiveness, metastasis, angiogenesis, and modifications in cell morphology and biochemistry (Vogelstein and Kinzler 1993). Chemical carcinogenesis may be induced by two groups of compounds: 1) genotoxic carcinogens that can interact with DNA, and 2) non-genotoxic carcinogens which have been shown to alter/induce tumour promotion, without interacting with DNA, via for example endocrine modification, immune suppression, and/or tissue-specific toxicity and inflammatory responses (Melnick et al. 1996; Williams 2001).

Cancer risk assessment has a qualitative (hazard identification) and a quantitative (dose-response analysis) component. Genotoxicity assays are currently used for the hazard identification of large numbers of chemicals for possible carcinogenic properties. Normally, genotoxicity tests are applied according to a (tiered) test strategy whereby a positive result triggers further testing and an *in vitro* positive result needs *in vivo* confirmation. A substance is suspected to be carcinogenic based on the results of *in vitro* and *in vivo* genotoxicity tests and a cancer bioassay is performed to obtain hazard confirmation and information with regards to cancer potency (*i.e.* dose-response analysis). Thus, since the genotoxicity tests are used as decisive predictive instruments, the performance of *in vitro* and *in vivo* genotoxicity tests in predicting the carcinogenic potential of chemicals is very important.

### 2.1 Genotoxicity testing strategy

A general strategy of short-term *in vitro* and *in vivo* genotoxicity assays for the hazard identification of potential genotoxic compounds is currently available. The standard genotoxicity testing strategy generally consists of a bacterial gene mutation assay, an *in vitro* mammalian mutation and/or a chromosome damage assay (Muller et al. 1999). An *in vivo* micronucleus test should be performed if the compound is found to induce chromosomal aberrations *in vitro*. Alternatively, if there are indications that the compound induces gene mutations *in vitro*, then the transgenic rodent mutation assay or the comet assay in potential target tissues should be performed (Eastmond et al. 2009). In general, an *in vivo* genotoxicity test involves a 1-4 day exposure to a chemical using 5-10 animals per dose with an average of 3 doses plus vehicle control. Only the transgenic rodent mutation assay consists of a 28-day exposure.

### 2.2 Cancer bioassay

The cancer bioassay is used for attaining information about the carcinogenic potency of chemicals by performing a dose-response analysis. This generally involves a 2-year exposure to a chemical using 50 animals per dose per sex with a minimal of 3 doses, in addition to the vehicle control for 2 species (OECD 2008). Thus, each cancer bioassay requires at least 800 animals for each substance. The majority of cancer bioassays are performed in mice and rats, although other species can be used if needed. Animals can be exposed via three

main administration routes: oral, dermal and inhalation. The administration time can vary from 18-24 months, depending on the animal species, which correlates to the major part of their life span and thereby takes into account the latency period of carcinogenesis. The highest dose is selected from a repeated dose 28-day and/or 90-day toxicity study that is performed prior to the carcinogenicity studies to derive the maximum tolerated dose.

## 3 Toxicogenomics

Genomics emerged more than a decade ago concurrently with the sequencing of the human genome. Genomic, or 'omics', technologies allow for a detailed description of biological systems as completely as possible at the level of mRNA, proteins, or metabolites. The application of genomics technologies in toxicology is termed toxicogenomics (Nuwaysir et al. 1999). It is a field of science that deals with the collection, interpretation, and storage of information about gene and protein activity, even within a single cell or tissue of an organism in response to chemicals. Toxicogenomics combines genetics, genomic-scale mRNA expression (transcriptomics), cell- and tissue-wide protein expression (proteomics), metabolite profiling (metabolomics) and bioinformatics with conventional toxicology in an effort to understand the role of gene-environment interactions in effect and disease, respectively (NAP 2005; Hamadeh and Afshari 2004; Oberemm et al. 2005). Generally, toxicogenomics is applied in two areas: a) to study mechanisms underlying toxicity; and b) to derive molecular expression patterns (*i.e.* molecular biomarkers) that predict toxicity or the genetic susceptibility to it.

### 3.1 Mechanism of action

Data on the mechanism or mode of action of a chemical have several applications in health risk assessments. The key issues that mechanistic data can inform in hazard identification include the relative sensitivity of humans to effects observed in animals, and human inter-individual variability and susceptibility to effects (including the identification of sensitive subpopulations). Mechanistic data are also used to provide information on the dose-response relationship, especially to support arguments regarding the expected existence or absence of thresholds at low doses. Since toxicity coincides with changes in mRNAs, proteins, and metabolites, toxicogenomics data can lead to the identification of the mode of action of chemicals. As such, it can lead to advances in human and ecological risk assessments of chemicals (Guyton et al. 2009).

When applied to the study of large classes of chemicals, toxicogenomic information can be used to define modes or mechanisms of toxic action. This greatly enhances the hazard identification processes when screening unknown chemicals. Comparing the expression profile of a chemical with unknown effects to the profiles of compounds with known effects allows faster and more accurate categorization into defined classes according to their mechanism of action (NRC 2007b). Several examples of this approach have been described in literature for various toxicity endpoints (Ellinger-Ziegelbauer et al. 2005; Fielden et al. 2007; Hamadeh et al. 2002a; Hamadeh et al. 2002b; Yuan et al. 2010). This approach requires large gene expression profile databases, which can be queried to help classify unknown compounds from exposed tissues without a detailed understanding of affected genes or biologic pathways.

### 3.2 Predicting toxicity

The most promising application of toxicogenomics involves the screening and prioritization of chemicals and drug candidates that warrant further development and testing. Predictive toxicogenomics uses transcriptome (or proteome or metabolome) data to predict a toxicological outcome, such as carcinogenicity. The goal is to identify a set of informative genes (biomarkers) that can detect or even predict possible adverse effects for new compounds at early stages, preferably after exposure to low dose levels (Kienhuis et al. 2010; NRC 2007a). For this, a large database of reference expression signatures of well-studied chemicals with known toxicity endpoints is required. The underlying assumption is that compounds eliciting similar toxic effects will elicit similar effects on gene

expression. Careful comparison of the expression profiles identifies genes whose patterns of expression can be used to distinguish the various groups of chemicals under analysis. These biomarkers are used to assign a compound to a particular group.

Several research groups, reviewed in Waters et al. (2010), have recently identified cancer-relevant gene sets that appear to discriminate carcinogens versus non-carcinogenic compounds. In general, the strategy involves selecting a training set of known carcinogenic and non-carcinogenic compounds.

Subsequently, exposure studies are performed, either *in vivo* or *in vitro*.

Toxicogenomic studies are performed in target organs or cells, and statistical classification approaches are used to identify genes that discriminate possible outcome categories. The training set genes are then used in classifying untested chemicals. Due to the enormous effort and high costs that coincide with the development of these databases, the largest and most highly standardized databases are only commercially available from private companies provided in a proprietary model to the pharmaceutical and biotechnology industry (Ganter et al. 2006).

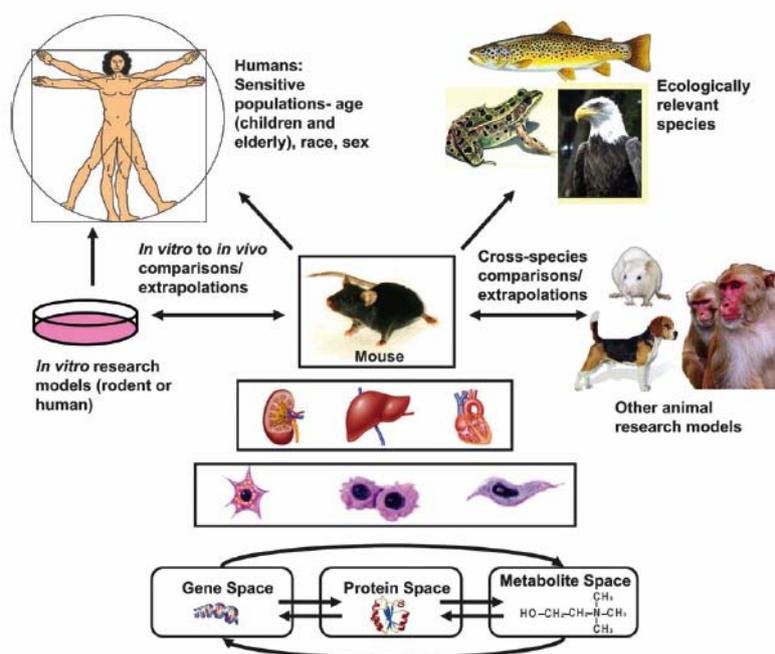


Figure 1. Toxicogenomics, the combination of genomics, proteomics, and metabolomics, allows for the examination of toxicant-induced alterations in biochemical networks which involves perturbations in gene, protein, and metabolite space. (From Boverhof and Zacharewski 2006)

Ideally, these biomarkers will be mechanistically-based and causally associated with the adverse effect. However, this is not necessarily the case. Moving from class prediction to mechanistic understanding often relies on additional work to translate toxicogenomic-based hypotheses to validated findings. Most toxic responses likely involve complex interactions between genes, proteins, and metabolites. The emergence of toxicogenomics provides the opportunity to simultaneously interrogate the broad molecular status of an organism, tissue, or cell experiencing toxicity within its gene, protein, and metabolite domains (see Figure 1). These data can be phenotypically anchored to toxicity observed at the

cellular and/or tissue level in a model organism to provide insights into the toxic mechanism of action. Comparison of these data across in vivo and in vitro research models allows for the identification of conserved responses which can be used to identify predictive biomarkers of exposure or toxicity and reduce the uncertainties in understanding the risk posed to humans and environmentally relevant species (Boverhof and Zacharewski 2006).

## 4 Toxicogenomics in carcinogenicity hazard assessment

Toxicogenomics has seen a significant investment in the pharmaceutical industry for both predictive and mechanism-based toxicology in an effort to identify candidate drugs more quickly and economically (Lesko and Woodcock 2004; Lord 2004; Yang et al. 2004). In contrast, chemical and agrochemical sectors have been less receptive to the implementation of toxicogenomics due to its questionable benefits in supporting risk assessment. Fundamental differences in drug versus environmental safety and risk assessment may be a factor contributing to the predominant use of toxicogenomics in the pharmaceutical sector. Despite significant progress in its development and implementation, deciphering meaningful and useful biological information from toxicogenomic data remains challenging for toxicologists and risk assessors.

In this section, we will explore where in the current test strategy for determining the carcinogenic potential of chemicals (see Figure 2) implementation of toxicogenomics might result in a significant improvement. The problems associated with *in vitro* and *in vivo* genotoxicity tests and the cancer bioassay will be discussed as well as whether toxicogenomic-based screens can help to address these.

### 4.1 Toxicogenomics in genotoxicity testing

#### 4.1.1 Possible applications of toxicogenomics in *in vitro* genotoxicity tests

*In vitro* genotoxicity tests are designed to measure the genotoxic potential of chemicals in bacteria or mammalian cells (see Figure 2) (Muller et al. 1999). Genotoxicity tests are used because of the strong association between the accumulation of genetic damage and carcinogenesis. Shortcomings of *in vitro* genotoxicity studies include the high proportion of non-carcinogenic chemicals that give positive results. Studies with large numbers of marketed pharmaceuticals have shown that approximately 50% of the non-carcinogens had positive genotoxicity findings (Snyder and Green 2001). From a risk assessment perspective, the fact that genotoxicity tests are overpredictive does not pose a problem, since this is a conservative, 'overprotective', approach. Even if toxicogenomics would have a better performance in predicting the carcinogenic potential, it is unlikely that these *in vitro* genotoxicity tests will be replaced by toxicogenomic-based screens given the high cost associated with them. Traditional *in vitro* genotoxicity tests provide a fast and cost-effective manner for detecting genotoxic compounds. From an economical standpoint, however, a positive *in vitro* genotoxicity result for a non-carcinogenic chemical, *i.e.* a cosmetic product, would imply a possible ban of the use of that substance when it is not allowed to verify the positive genotoxicity result. Only in this instance, the use of toxicogenomic-based screens can have an added value.

#### 4.1.2 Possible applications of toxicogenomics in *in vivo* genotoxicity tests

*In vivo* genotoxicity tests are performed when there is a positive result in any *in vitro* genotoxicity test (Figure 2). The choice of follow-up *in vivo* tests should depend on the type of genotoxic damage detected from *in vitro* tests (*i.e.* gene mutations or chromosomal aberrations). An *in vivo* micronucleus test should be performed if the compound is found to induce chromosomal mutations *in vitro*. If there are indications that the compound induces gene mutations *in vitro*, then the transgenic rodent mutation assay or the comet assay in potential target tissues should be performed (EC 2006). When the proper follow-up *in vivo* genotoxicity tests are performed, the carcinogenic potential of a chemical is predicted with a rather high accuracy (Kirkland and Speit 2008). So, from a risk assessment perspective, there is not really a problem that needs to be addressed. Implementation of toxicogenomics in *in vivo* genotoxicity testing contributes to a limited extent to alternatives to animal testing. The number of

animals required could be slightly reduced compared to the classical tests, *i.e.* by reducing the number of doses tested. In addition, refinement of animal experimentation could be achieved by reducing the dose(s) and the exposure duration. The latter mainly holds true when comparing a toxicogenomic-based approach with the transgenic rodent mutation assay.

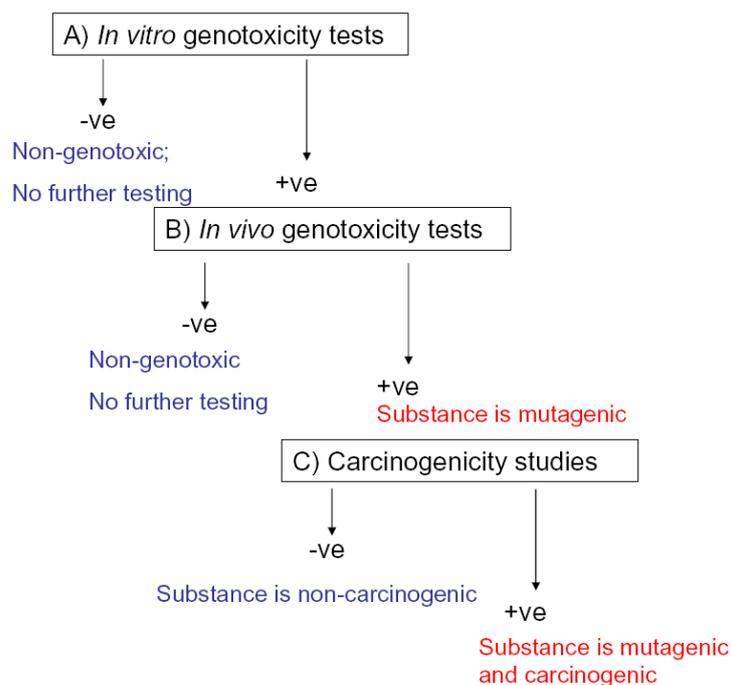


Figure 2. The current test strategy for mutagenicity and carcinogenicity testing for new and existing chemicals

#### 4.1.3 Example of toxicogenomics in genotoxicity hazard assessment

Toxicogenomics and statistical methods have progressed such that it may be possible to use them in acute or subchronic studies to predict toxicity. Several research groups have recently identified cancer-relevant gene sets that appear to discriminate carcinogens versus non-carcinogenic compounds (reviewed in Waters et al. (2010)). We tested the potential of toxicogenomics to predict genotoxicity using primary mouse hepatocytes. For this, gene expression profiles of primary mouse hepatocytes were generated after *in vitro* exposure to a chemical. The chemicals tested were of various classes: genotoxic carcinogens, non-genotoxic carcinogens, or non-carcinogens.

To analyze the gene expression profiles obtained, we performed principal component analysis (PCA). PCA involves a mathematical procedure that aims to group the gene expression profiles obtained based on the variation between the various profiles. For our study, this analysis resulted in a clear separation of the genotoxic carcinogenic compounds from the non-genotoxic compounds, irrespective whether these non-genotoxic substances are carcinogenic or not (see Figure 3).

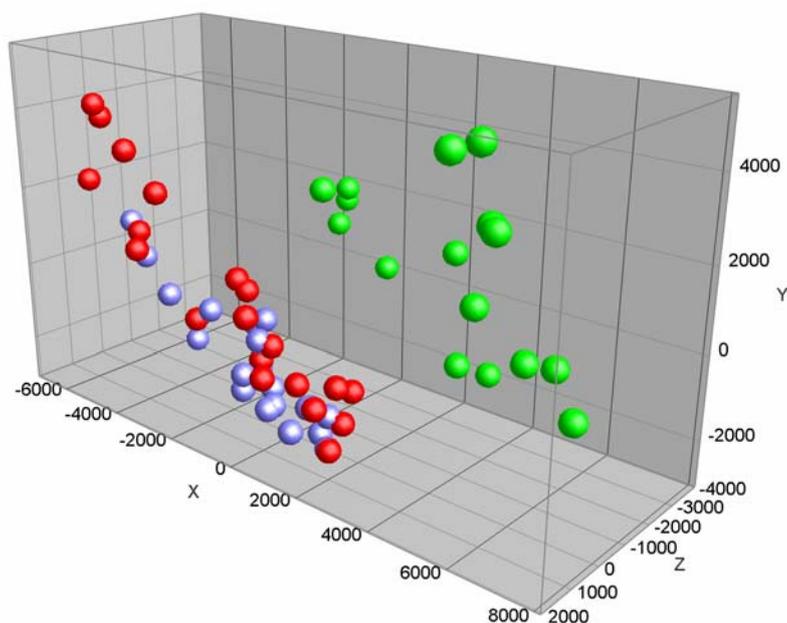


Figure 3. All probe sets on the array were used in the principal component analysis. The principle components explain 26%, 15.7% and 9.1% of the variance. The markers are coloured by class (green: genotoxic carcinogen, red: non-genotoxic carcinogen, blue: non carcinogen).

## 4.2 Cancer bioassay

Depending on the regulatory framework and the particular conditions, including positive results from genotoxicity tests, a lifetime cancer bioassay may be performed with the purpose of establishing if the compounds are indeed carcinogenic and to derive a point of departure for further risk assessment. Carcinogenicity studies are needed for the quantitative or dose-response analysis to determine the relationship between dose and the incidence of tumour formation (carcinogenic potency). The cancer bioassay has several drawbacks. First, the cancer bioassay uses a large number of animals. There is an increasing societal interest in lowering the amount of experimental animals for chemical testing, which pressures governments to tighten regulation. For instance, new policies in the European Union have prohibited the use of animals for chemical testing of cosmetics since March 2009 and a total ban on animal testing of cosmetics will come into effect in 2013 regardless of the availability of approved non-animal alternatives (EC 2008). Second, the time required for testing is long and the costs of a bioassay range from approximately one to several million euros, depending on route of exposure (Jacobson-Kram et al. 2004). Finally, the cancer bioassay does not provide any information about the mechanism of action of the compounds tested. Mechanistic information provides a better understanding of the human relevance of the effects observed in animals for humans (Guyton et al. 2009). For these reasons, short-term experimental approaches that can improve human cancer predictivity and/or provide information on the mode of action need to be developed.

As mentioned above, the purpose of the cancer bioassay is for dose-response assessment and to determine the relationship between dose and the incidence of tumour formation. The question remains whether toxicogenomics can be used in a quantitative manner, with fewer animals and reduced costs (see Figure 2). Toxicogenomic-based dose-response studies can provide better insights with

respect to low-dose effects and mode of action, because subtle changes at the molecular level that may lead to carcinogenesis can be detected (NRC 2005, 2007a). Promising results were obtained when toxicogenomic dose-response data were analyzed using an integrated approach which combined benchmark dose (BMD) calculations with gene ontology classification analysis (Thomas et al. 2007). This method was applied to gene expression data in the rat epithelium after formaldehyde exposure. The differentially expressed genes that showed a dose-response were identified. The average BMD and lowest confidence interval of the BMD (BMDL) were calculated for each category resulting in the identification of doses at which individual cellular processes were altered (Thomas et al. 2007). The mean BMD values for the gene expression changes were similar to those obtained when measuring changes in cell proliferation (cell labelling index), DNA damage responses (DNA or protein cross-links), and rat nasal tumour induction (Schlosser et al. 2003). This study is an example of molecular changes following chemical exposure and the derivation of potency estimates at which different cellular processes are altered. It is conceivable that with this integrated approach, potency estimates can be derived from short-term exposures thereby eliminating the need to perform carcinogenicity studies in the future.

Overall, toxicogenomic-based screens can have a significant impact in reducing the number and discomfort of animals used in carcinogenicity studies. In addition, toxicogenomics can be used to define mechanisms of action, improve interspecies extrapolations and maybe be utilized in a quantitative manner to derive potency estimates that are equally or more conservative than those derived from carcinogenicity studies. Clearly, more dose-response and time-course studies in large sets of compounds encompassing multiple toxicological endpoints are needed to fill the existing data gaps.

## 5 Toxicogenomics from a regulatory perspective

### 5.1 Requirements for implementation of toxicogenomics in risk assessment strategies

From a hazard and risk assessment perspective, toxicogenomics is no more or less than an effect analysis tool, like clinical biochemistry or histopathology, though be it a very advanced one. Nevertheless, for the results of toxicogenomics assessments, in order to be included into hazard or risk assessment strategies a number of steps still has to be taken, especially if studies are intended to replace already existing tests, e.g. for the purpose of reduction of test animal use.

First, an adequate study design needs to be established. A study design is very much dependent on the scientific question that the study is intended to address: a study which should only provide information on hazard (*i.e.* is the substance genotoxic and/or carcinogenic?) is designed differently from a study intended for risk assessment purposes (*i.e.* how potent is the substance? or how relevant is the observed effect in animals to humans?). While in the first set-up only a few dose levels need to be investigated up to a certain (high) dose level in which toxicity is observed, several dose levels need to be included to define the dose-response relationship and reliably derive a potency estimate. Therefore, for toxicogenomics to be incorporated into the current test strategy, scientists need to clearly address whether toxicogenomics is used for a) hazard identification (*i.e.* classification of substances), b) quantitative dose-response analysis and derivation of potency estimates, or c) evaluation of the human relevance. This distinction is necessary for regulatory acceptance. Common to all toxicological studies it is necessary to standardize study designs with respect to dose levels, the number of dose groups and the width of the dose interval. Highest dose levels should be chosen in a way that interference with parameters indicative for acute toxicity is avoided. Other design parameters which usually are standardized are number of animals/group (or number of tissue cultures), the exposure route (representative of human exposure routes), the exposure duration, the possible post-exposure interval to avoid loss of information due to down-regulation and time of sacrifice taking into account diurnal variability. Data obtained in non-exposed animals and spread in parameters should be limited to give a reproducible historical control with low background signals in relevant parameters to minimize problems with signal interpretation.

Second, the ability and reliability of a study to detect hazardous properties needs to be determined and compared to results from existing assays which follow harmonized test guidelines for the respective endpoint. In other words, specificity, sensitivity and predictivity should be determined in an extensive validation process. In this respect it is very important to determine what the test should actually predict. Within the scope of the current report, this means carcinogenicity or genotoxicity, and the validation process should be based on equivalent hazard comparisons (*i.e.* genotoxicity should preferably be validated against the results of genotoxicity tests). When tests are designed to provide information on hazard, it is relevant to establish to what extent absence of changes is 'proof' of absence of effects. This is of particular relevance if doubts can be raised when the study target has been exposed to the chemical (or an activated derivative/biotransformation product) and only one or a few tissues are analyzed. Two issues are raised with this scenario: a) is the absence of a measured response in one or few tissues indicative of the absence of an effect in all tissues? and b) can effects in vital or commonly assayed tissues such as the liver, lung or spleen provide relevant information indicative of toxic events in other tissues that are not evaluated? (*i.e.* can a study in liver provide data on toxic events in the brain?).

Third, toxicogenomic-based screens require some methodology-specific issues to be dealt with such as choice of platform (*i.e.* the type of array used), the genetic probes included in each platform, and the standardization of the analyzed set of signals per endpoint of interest (*i.e.* should the same set of gene expression signals be used for genotoxicity? or should a highly variable set differing from study to study be acceptable?). Depending on the platform, the parameters that define a toxicological 'response' need to be identified, *i.e.* the set of parameters that decide whether a pathway is down- or upregulated should be clearly established. In addition, the criteria that describe how changes are identified as being 'adverse' instead of merely adaptive need to be determined. This is especially important for studies intended to be used for risk-assessment.

Fourth, interpretation of study data maybe facilitated by the availability (and inclusion) of appropriate positive controls. Development of (a set of) positive controls may be very crucial. Again, with focus on genotoxicity and carcinogenicity, such positive controls should be informative of dependency of bioactivation (especially in *in vitro* studies) and they should be informative on possible genotoxic or non-genotoxic mechanism of action for development of carcinogenicity. However, for the latter, it may not be possible to identify one single or a few positive controls, since non-genotoxic carcinogens may differ considerably with respect to the way they interfere with biological systems and it may not be possible to find one common set of parameters which would be suitable to cover all possible non-genotoxic mechanisms. In addition, in order to avoid problems with transparency of data analysis, standardization of data-analysis methodology should be achieved. If not possible, then the methodology should be described in a way that interpretation of analysis results can be evaluated independently by the risk assessor and in any way it is essential that raw study data should be included to facilitate independent re-analysis of the information.

Overall, it is generally accepted that toxicogenomics can be a very powerful tool to unravel mechanisms of action. However, it is not by definition clear that a toxic response will only be elicited via a single mechanism of action. For example, it has been mentioned for acetaminophen that hepatocellular toxicity not only depends on loss of mitochondrial function and oxidative stress, but also inflammatory responses mediated through mononuclear cells, macrophages, Kupffer cells and neutrophils (Kienhuis et al. 2009). These cell types would not be present in hepatocyte cultures and therefore the dose-response (or rather concentration response) for such cultures might not be reflective of the dose-response in liver *in vivo*. Despite this, the unravelling of mechanisms of action, whether for genotoxicity, carcinogenicity or any other toxic response can be very helpful to support extrapolation processes, in particular when comparing *in vivo* animal tissues to cells *in vitro*, or when comparing animal data to data from human tissues or cells. However, since toxicity-testing in human-derived material is not common (*i.e.* data from human tissues are very limited and only a few substances have data from human tissues), such supporting information is often of limited value in animal-to-human extrapolations.

Finally, additional considerations need to be kept in mind when analyzing *in vitro* studies, not only with respect to the relevance of the (combination of) cell type(s) studied (see also the paragraph above), but also with respect to exposure: in many *in vitro* studies little if any attention has been paid to the actual exposure of the cells. Usually only nominal concentrations are given. It can be questioned how exposure in such systems should be defined: as (measured) protein-bound or unbound concentration in the medium or as concentration within the cell. Also medium concentrations are assumed to be constant in time. Although this may not be true, the time course of a substance concentration in *in vitro* studies will always be different from the time course of plasma levels *in vivo*, and extrapolation of concentration-effect data from *in vitro* studies to *in vivo* exposures would require inclusion of adequate

toxicokinetic modelling to take this into account. An extensive discussion on this issue can be found in Blaauboer (2010).

## **5.2 What should be done to satisfy regulators and successfully implement toxicogenomics in risk assessment?**

### *5.2.1 Standardization*

Toxicogenomic technologies have not yet been fully developed to be utilized to support risk decisions. Several recommendations have been provided by the U.S. National Research Council's Committee on the requirements needed for toxicogenomics to be used as a tool to better the current cancer risk assessment including:

- the development of specialized bioinformatics, statistical and computational tools to analyze toxicogenomic data
- the generation of a national data resource that is open to the research community to encourage data standardization,
- the development of algorithms to easily identify orthologous genes and proteins in various species, of tools to integrate data across multiple analytical platforms, and of computational models to enable the study of network responses and systems-level analyses,
- the standardization of genome annotation, and
- the development of standards to ensure data quality (NRC 2005, 2007a).

These requirements will help to validate toxicogenomic-based screens and provide evidence on whether these screens can better discriminate between carcinogens and non-carcinogens than the traditional tests.

### *5.2.2 Quantitative aspects*

Worldwide toxicogenomics is predominantly used as a tool for the elucidation of a mechanism of action and thus for hazard identification of a chemical. Irrespective of the toxicological endpoint, information on both mechanisms of action and dose-response relationships form the basis for any toxicological risk assessment. Despite all the efforts and all the information obtained, toxicogenomics is still not ready as a tool for human toxicological risk assessment. This is mainly due to the fact that only very few studies have focused on dose-response relationships at gene expression level. In order to integrate toxicogenomic endpoints in quantitative risk assessment it is essential to know whether relevant changes in gene expression occur in a dose-dependent manner and how these changes should be interpreted, *i.e.* to know at which levels of exposure changes in gene expression represent adverse effects. Figure 4 may illustrate this. At the lower exposures, treatment will result in a change of gene expression profile. However after discontinuing the treatment the gene expression profile will return to the profile at the start of treatment.

At a specific dose, the gene expression profile will also change but never return to the starting profile. Yet the increase is under homeostatic control and the effect is not adverse. This dose (see Figure 4, point A) represents the point where homeostasis changes into an adaptive response. Finally there will be a dose where the gene expression profile is no longer under the homeostatic control resulting in a toxicological effect. This dose (see Figure 4, point B) represents the point where the effects change from an adaptive response into a toxic, adverse response.

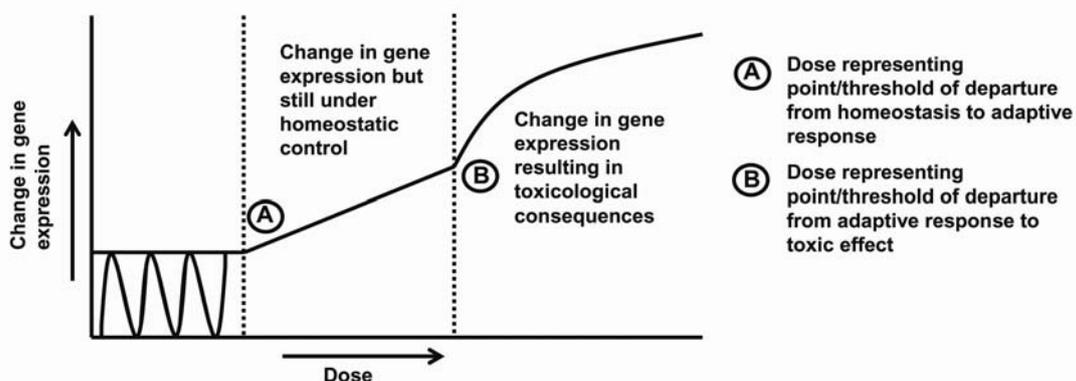


Figure 4. Steps in progression of changes in gene expression towards an adverse response

To recognize the doses A and B which are the relevant points of departure for the estimation of (extrapolation towards) reference values *i.e.* acute reference dose or for chronic exposures the acceptable daily intake or tolerable daily intake, it is essential to know the process of adversity of the compound under investigation. Only with that knowledge, translation of toxicogenomic data into risk assessment will be possible. Data should be collected for substances for which the mechanism of action is known and for which relevant (histopathological) data at various time points during carcinogenesis are available. The findings of the toxicogenomic studies at the different doses can be compared with relevant (histopathological) changes enabling us to determine point A and B and to decide where observed effects actually become adverse.

## 6 Conclusions

Toxicogenomics may help improve the current test strategy for carcinogenicity. The added value of toxicogenomics applied in genotoxicity testing is limited, since the current tests are rather overprotective. In carcinogenicity testing, however, implementation of toxicogenomics may have a significant impact. It could be used to define mechanisms of action, which is particularly important for non-genotoxic carcinogens, and improve interspecies extrapolations. It can also be utilized in a quantitative manner to derive potency estimates. In addition, implementation of toxicogenomics contributes to alternatives to animal testing by reducing the number and discomfort of animals used in carcinogenicity studies. In order to successfully integrate toxicogenomic endpoints in quantitative risk assessment, however, more studies investigating dose-response relationships for large sets of compounds at the gene expression level are needed to learn whether relevant changes in gene expression occur in a dose-dependent manner and how these changes should be interpreted in terms of adversity. In addition, (worldwide) standardization of several aspects of toxicogenomics is needed in order to be utilized in risk assessment.

## References

- Blaauboer, B. J. 2010. Biokinetic modeling and in vitro-in vivo extrapolations. *J Toxicol Environ Health B Crit Rev* 13 (2-4):242-252.
- Boverhof, D. R. and T. R. Zacharewski. 2006. Toxicogenomics in risk assessment: applications and needs. *Toxicol Sci* 89 (2):352-360.
- Eastmond, D. A., A. Hartwig, D. Anderson, W. A. Anwar, M. C. Cimino, I. Dobrev, G. R. Douglas, T. Nohmi, D. H. Phillips, and C. Vickers. 2009. Mutagenicity testing for chemical risk assessment: update of the WHO/IPCS Harmonized Scheme. *Mutagenesis* 24 (4):341-349.
- EC. 2003. Technical guidance document on risk assessment in support of Commission Directive 93/67 EEC on risk assessment for new notified substances and Commission Regulation (EC) No. 1488/94 on risk assessment for existing substances. European Commission (EC) (Internet publication at <http://ecb.jrc.it>) Part I.
- EC. 2006. Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directive 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC.
- EC. 2008. EC Consolidated version of Cosmetics Directive 76/768/EEC. European Commission (EC) (Internet publication at <http://ecb.jrc.it>).
- Ellinger-Ziegelbauer, H., B. Stuart, B. Wahle, W. Bomann, and H. J. Ahr. 2005. Comparison of the expression profiles induced by genotoxic and nongenotoxic carcinogens in rat liver. *Mutat Res* 575 (1-2):61-84.
- Fielden, M. R., R. Brennan, and J. Gollub. 2007. A gene expression biomarker provides early prediction and mechanistic assessment of hepatic tumor induction by nongenotoxic chemicals. *Toxicol Sci* 99 (1):90-100.
- Ganter, B., R. D. Snyder, D. N. Halbert, and M. D. Lee. 2006. Toxicogenomics in drug discovery and development: mechanistic analysis of compound/class-dependent effects using the DrugMatrix database. *Pharmacogenomics* 7 (7):1025-1044.
- Guyton, K. Z., A. D. Kyle, J. Aubrecht, V. J. Cogliano, D. A. Eastmond, M. Jackson, N. Keshava, M. S. Sandy, B. Sonawane, L. Zhang, M. D. Waters, and M. T. Smith. 2009. Improving prediction of chemical carcinogenicity by considering multiple mechanisms and applying toxicogenomic approaches. *Mutat Res* 681 (2-3):230-240.
- Hamadeh, H. K. and C. Afshari. 2004. *Toxicogenomics: Principles and Applications*. Hoboken, NJ: Wiley-Liss ISBN 0-471-43417-5.
- Hamadeh, H. K., P. R. Bushel, S. Jayadev, O. DiSorbo, L. Bennett, L. Li, R. Tennant, R. Stoll, J. C. Barrett, R. S. Paules, K. Blanchard, and C. A. Afshari. 2002a. Prediction of compound signature using high density gene expression profiling. *Toxicol Sci* 67 (2):232-240.
- Hamadeh, H. K., P. R. Bushel, S. Jayadev, K. Martin, O. DiSorbo, S. Sieber, L. Bennett, R. Tennant, R. Stoll, J. C. Barrett, K. Blanchard, R. S. Paules, and C. A. Afshari. 2002b. Gene expression analysis reveals chemical-specific profiles. *Toxicol Sci* 67 (2):219-231.

- IARC. 2008. World Cancer Report, <[http://www.iarc.fr/en/publications/pdfs-online/wcr/2008/wcr\\_2008.pdf](http://www.iarc.fr/en/publications/pdfs-online/wcr/2008/wcr_2008.pdf)>
- Jacobson-Kram, D., F. D. Sistare, and A. C. Jacobs. 2004. Use of transgenic mice in carcinogenicity hazard assessment. *Toxicol Pathol* 32 Suppl 1:49-52.
- Kienhuis, A. S., J. G. Bessems, J. L. Pennings, M. Driessen, M. Luijten, J. H. van Delft, A. A. Peijnenburg, and L. T. der Ven. 2010. Application of toxicogenomics in hepatic systems toxicology for risk assessment: Acetaminophen as a case study. *Toxicol Appl Pharmacol* 21:21.
- Kienhuis, A. S., M. C. van de Poll, H. Wortelboer, M. van Herwijnen, R. Gottschalk, C. H. Dejong, A. Boorsma, R. S. Paules, J. C. Kleinjans, R. H. Stierum, and J. H. van Delft. 2009. Parallelogram approach using rat-human in vitro and rat in vivo toxicogenomics predicts acetaminophen-induced hepatotoxicity in humans. *Toxicol Sci* 107 (2):544-552.
- Kirkland, D. and G. Speit. 2008. Evaluation of the ability of a battery of three in vitro genotoxicity tests to discriminate rodent carcinogens and non-carcinogens III. Appropriate follow-up testing in vivo. *Mutat Res* 654 (2):114-132.
- Lesko, L. J. and J. Woodcock. 2004. Translation of pharmacogenomics and pharmacogenetics: a regulatory perspective. *Nat Rev Drug Discov* 3 (9):763-769.
- Lord, P. G. 2004. Progress in applying genomics in drug development. *Toxicol Lett* 149 (1-3):371-375.
- Melnick, R. L., M. C. Kohn, and C. J. Portier. 1996. Implications for risk assessment of suggested nongenotoxic mechanisms of chemical carcinogenesis. *Environ Health Perspect* 104 Suppl 1:123-134.
- Muller, L., Y. Kikuchi, G. Probst, L. Schechtman, H. Shimada, T. Sofuni, and D. Tweats. 1999. ICH-harmonised guidances on genotoxicity testing of pharmaceuticals: evolution, reasoning and impact. *Mutat Res* 436 (3):195-225.
- NAP. 2005. The National Academies Press: Communicating Toxicogenomics Information to Nonexperts: A workshop summary
- NRC. 2005. National Research Council: Toxicogenomic Technologies and Risk Assessment of Environmental Carcinogens: A workshop summary. National Research Council (US) Committee on How Toxicogenomics Could Inform Critical Issues in Carcinogenic Risk Assessment of Environmental Chemicals. Washington (DC): National Academies Press (US); 2005. The National Academies Collection: Reports funded by National Institutes of Health.
- NRC. 2007a. National Research Council (US) Committee on Applications of Toxicogenomic Technologies to Predictive Toxicology. Applications of Toxicogenomic Technologies to Predictive Toxicology and Risk Assessment. The National Academies Collection: Reports funded by National Institutes of Health.
- NRC. 2007b. National Research Council. Applications of Toxicogenomic Technologies to Predictive Toxicology and Risk Assessment.
- Nuwaysir, E. F., M. Bittner, J. Trent, J. C. Barrett, and C. A. Afshari. 1999. Microarrays and toxicology: the advent of toxicogenomics. *Mol Carcinog* 24 (3):153-159.
- Oberemm, A., L. Onyon, and U. Gundert-Remy. 2005. How can toxicogenomics inform risk assessment? *Toxicol Appl Pharmacol* 207 (2 Suppl):592-598.
- OECD. 2008. Draft OECD guideline for the testing of chemicals, Test Guideline 451: Carcinogenicity studies. <http://www.oecd.org/dataoecd/30/46/41753121.pdf>.

- Schlosser, P. M., P. D. Lilly, R. B. Conolly, D. B. Janszen, and J. S. Kimbell. 2003. Benchmark Dose Risk Assessment for Formaldehyde Using Airflow Modeling and a Single-Compartment, DNA-Protein Cross-Link Dosimetry Model to Estimate Human Equivalent Doses. *Risk Analysis* 23 (3):473-487.
- Snyder, R. D. and J. W. Green. 2001. A review of the genotoxicity of marketed pharmaceuticals. *Mutat Res* 488 (2):151-169.
- Thomas, R. S., B. C. Allen, A. Nong, L. Yang, E. Bermudez, H. J. Clewell, 3rd, and M. E. Andersen. 2007. A method to integrate benchmark dose estimates with genomic data to assess the functional effects of chemical exposure. *Toxicol Sci* 98 (1):240-248.
- Vogelstein, B. and K. W. Kinzler. 1993. The multistep nature of cancer. *Trends Genet* 9 (4):138-141.
- Waters, M. D., M. Jackson, and I. Lea. 2010. Characterizing and predicting carcinogenicity and mode of action using conventional and toxicogenomics methods. *Mutat Res* 23:23.
- Williams, G. M. 2001. Mechanisms of chemical carcinogenesis and application to human cancer risk assessment. *Toxicology* 166 (1-2):3-10.
- Yang, Y., E. A. Blomme, and J. F. Waring. 2004. Toxicogenomics in drug discovery: from preclinical studies to clinical trials. *Chem Biol Interact* 150 (1):71-85.
- Yuan, X., M. J. Jonker, J. de Wilde, A. Verhoef, F. R. Wittink, J. van Benthem, J. G. Bessems, B. C. Hakkert, R. V. Kuiper, H. van Steeg, T. M. Breit, and M. Luijten. 2010. Finding maximal transcriptome differences between reprotoxic and non-reprotoxic phthalate responses in rat testis. *J Appl Toxicol* 9 (10).

