The development of improved and new in vitro assays for detecting the genotoxic and non-genotoxic carcinogenic potential of chemicals in the discovery phase of drug development

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Table of contents

Chapter 1

General introduction

Introduction

In drug development, toxicity is an important factor for attrition, resulting in a failure rate of 30%-40%. Hepatotoxicity, nephrotoxicity, cardiovascular safety, reproduction toxicity, developmental toxicity (teratogenicity), genotoxicity and carcinogenicity are the main causes for attrition in safety assessment.

Screening on these aspects in the early discovery phase of drug development and using these data for compound optimization and deselection might result in drug development candidates with an improved success rate. The present thesis focuses on early screening for genotoxicity and carcinogenicity.

In recent years some progress has been made with assays to assess genotoxicity and non-genotoxic carcinogenicity at the end of the discovery phase. However, the time point at which these genotoxicity and carcinogenicity assays are performed is still relatively late, only a few assays for such a strategy are available, the throughput of these assays is in general still low and most of them have not yet been validated extensively. An additional drawback is that the currently used in vitro assays for the detection of genotoxicity give a high rate of false positive results, which makes application in the early discovery phase of drug development cumbersome.

The goal of this thesis is therefore to develop improved and new in vitro assays for detecting the genotoxic and non-genotoxic carcinogenic potential of chemicals, validate these assays with proper reference compounds, and to develop a strategy for application of these assays in the early discovery phase of drug development.

The drug developmental process and drug attrition

The costs spend on R&D have increased tremendously during the last decades. In spite of the increase in R&D expenses, the development of new assay methods, new techniques in liquid handling, robotics, analytical tools and software, the yearly number of approved new drugs has declined [1].

The process of drug development is shown in Figure 1 and can be divided into discovery, exploratory development, and the full development and launch of the drug. In the discovery phase biological targets are validated and high-throughput screening is used to find molecules that interact with these targets (hits). After identification of the most promising hit (lead molecule), this molecule is further optimized (lead optimization) to a compound that shows pharmacological activity in an animal model. This first phase that ends with delivering a development candidate takes around 3-4 years.

The second, exploratory development phase, consists of preclinical development and

first into man studies (FIM). In vitro and in vivo testing is performed to assess the safety of the compound. Assays to show genotoxic potential of compounds are also performed in this phase. When this first set of regulatory assays shows no serious adverse effects an investigational new drug application (IND) is filed to the regulatory authorities such as the Food and Drug Administration (FDA) and European Medicines Agency (EMA). Then FIM phase I clinical studies can be initiated, to study the safety and pharmacodynamics of the compound in healthy humans. Successful completion of this second phase results in the acquirement of a statement of no objection (SNOB). The process from development candidate to SNOB takes again around 3-4 years.

In the third phase large clinical studies (phase II, III) and complex in vivo animal studies like carcinogenicity testing are performed. Results that indicate the absence of adverse effects can lead to the delivery of a full development candidate (FDC) which after positive review by regulatory authorities will result in launch of a new prescription drug. This third phase takes around 6-8 years and is the most expensive part of drug development.

Thus the complete process of developing a new drug takes approximately 12-16 years. The average developmental costs of a new prescription drug are high and estimated at approximately 800 million USD [2]. This figure does not take into account the costs of failed drugs as there is a high attrition rate of 90%. When this high attrition rate is taken into account the costs of developing one new drug can go up to 1.5 billion.

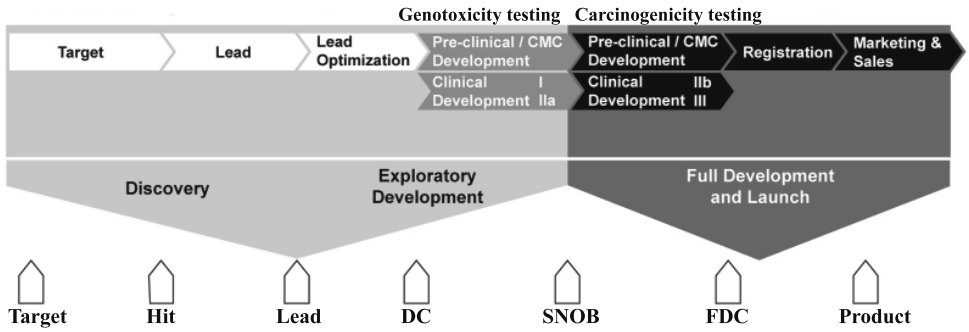

Figure 1. Overview of the drug development process. The development of a new prescription drug takes around 12-16 years. The first phase that takes around 3-4 years consist of target validation, hit/lead finding and lead optimization and ends with the selection of a development candidate (DC). The second phase consists of preclinical safety studies and the first into man clinical studies. This phase ends after around 3-4 years with a statement of no objection (SNOB). In the third phase that takes around 6-8 years large clinical studies and complex preclinical studies are performed. This phase can deliver a full development candidate (FDC) and after review and registration result in market launch of a new prescription drug. Genotoxicity and carcinogenicity testing is respectively performed during exploratory and full development.

Table 1. Percentage of failure for toxicity reasons of compounds that were developed at Roche, Merck&BMS and the legacy NV Organon between 1960 and 2000 [4,5]. The most important toxicities issues for attrition are indicated in **bold** (Σx =12).

Toxicity failure	Roche	Merck & BMS	NV Organon
Genotoxicity/carcinogenicity	6	8	**20**
Reproductive toxicity	2	5	**20**
Hepatotoxicity	**20**	15	12
Cardiovascular safety	16	**27**	12
Skin toxicity	10	-	x
Central nervous system side effects	10	7	x
Blood toxicity	6	7	x
Renal toxicity	4	2	12
Gastrointestinal toxicity	4	3	x

Approximately 30%-40% of the new drug candidates fail in the developmental phase due to toxicological side effects [1, 3]. Several unpredicted toxicities account for the failure of new chemical entities (NCEs). Main causes are hepatotoxicity, nephrotoxicity, cardiovascular safety, reproduction toxicity and developmental toxicity (teratogenicity), and genotoxicity/carcinogenicity (Table 1). The percentage of compounds that fails on each of these endpoints depends on the portfolio of disease related compound classes that a pharmaceutical company works on. Depending on the company, the detection of compounds with genotoxic or carcinogenic potential accounted for 6-20% of the drug failures. Prescreening on these aspects before the selection of a development candidate may therefore result in drug development candidates with an improved success rate.

Genotoxicity

Genotoxic compounds (genotoxicants) are compounds that cause mutations in the DNA. Mutations are defined as permanent changes in the DNA. Three types of mutations can be distinguished. Small changes in the DNA at the level of bases are the so-called single-point mutations or gene mutations. These small mutations can result in base-pair substitutions, deletions or addition of bases, the latter two resulting in so-called frameshift mutations. Moreover, there are two types of mutations that occur at the level of the chromosomes. Structural chromosomal aberrations are major changes in the structure of the DNA. These changes are due to breakages, deletions, exchanges, or rearrangements of DNA. Compounds that cause structural chromosomal changes are called clastogens. The third group of mutations are changes in the number of chromosomes. Such changes are called aneuploidy and are caused by aneugenic compounds. Compounds can cause

one or more types of mutations.

Genotoxic compounds can be sub-classified into compounds that are DNA reactive themselves (i.e. compounds that form DNA adducts and DNA cross-linkers), and compounds that are non-DNA reactive but react with certain proteins in the cell, like for example compounds that inhibit topoisomerase enzymes, and thereby cause DNA damage in an indirect way. Moreover, a lot of genotoxic compounds need to be activated metabolically before they show there genotoxic mode of action. Such compounds are called proximate genotoxicants or carcinogens.

Carcinogenicity

Carcinogens are compounds that induce the incidence of tumor formation. Carcinogenic compounds can have a genotoxic or non-genotoxic mode of action. Two models lay the foundation for the description of tumor development (carcinogenesis). These models are the multistage model of Armitage and Doll [6] and the initiation promotion model of Berenblum [7]. It is assumed that the development of tumors is a multistage process in which several mutations are needed for the transformation of a normal cell into an autonomic growing cell (neoplastic cell). The first phase (initiation phase) starts with DNA damage that is caused by endogenous factors or exogenous factors like for example DNA reactive or indirect genotoxic compounds.

Besides the initiation phase in which the mutations are irreversibly fixated in the DNA, there is a second phase, the promotion phase, in which the neoplastic cell develops into an observable and clinically manifest tumor. The third phase in the development of a tumor is the progression phase. This involves growth of the tumor, invasion into the surrounding tissue and metastasis.

Non-genotoxic carcinogens can induce tumor formation by several mechanisms [8]. These mechanisms may include receptor mediated induction/stimulation of carcinogenesis, cytotoxicity, endocrine modification, immunosuppression, inflammation, oxidative stress, hyper-/hypo-methylation and inhibition of gap-junction mediated intercellular communications. Especially the receptor mediated induction/stimulation is responsible for several forms of rodent specific carcinogenicity. Examples are species specific activation of the aryl hydrocarbon receptor (AhR), the peroxisome proliferator activated receptor alpha (PPARα) and the constitutive androstane receptor (CAR) [8]. Assays that measure the activation of these receptors in rodent and human cells in the early phase of drug development might be useful for early detection of non-genotoxic carcinogens.

Regulatory tests to detect compounds with genotoxic and carcinogenic potential

Regulatory tests to detect genotoxic potential

A stepwise approach (tiered approach) is applied in regulatory genotoxicity testing [9]. In vitro assays with a high sensitivity are used as a first step to see whether the test compounds have intrinsic genotoxic activity. These tests are then followed by in vivo tests that are designed to assess the relevance of the in vitro result for the in vivo situation. In vivo genotoxicity studies are also performed for the reason that some genotoxicants are only detected in vivo [10]. A decision tree for regulatory genotoxicity testing including a description of necessary follow up testing is described in detail later in this chapter.

Genotoxicity testing is in comparison to regulatory testing of carcinogenicity relatively cheap and fast. Compounds without genotoxic liability can proceed to FIM clinical trials. The carcinogenic potential is further assessed in the full developmental phase of drug development.

The regulatory test strategy consists of a battery of tests because the three types of genotoxicity (gene mutations, clastogenicity and aneugenicity) cannot be detected by a single test. The standard test battery required for genotoxicity testing is described in ICH guideline S2B for the registration of pharmaceuticals for human use and consists of (1) the Ames assay to detect gene mutations in bacteria, (2) an in vitro chromosome aberration or mouse lymphoma TK assay in mammalian cells and (3) an in vivo chromosome damage assay (chromosome aberration or micronucleus assay). Specific technical aspects of these regulatory tests are described in ICH guideline S2A for the registration of pharmaceuticals for human use.

The assays from the standard test battery have a different sensitivity, specificity and predictivity for carcinogenicity. The performance definitions are shown in Table 2. For the calculations of the sensitivity, the results of the genotoxicity assays are compared with the results of the carcinogenicity tests. It is however important to note that several of the carcinogenic compounds act via a non-genotoxic mode of action. Genotoxicity tests will thus never reach a sensitivity of 100% for carcinogenicity. The assays from the regulatory genotoxicity test battery are described in more detail in the next sections and the performances scores are shown in Table 3.

Table 2. Performance definitions for genotoxicity tests.

Term	Definition
Sensitivity	Percentage of carcinogens positive in the test
Specificity	Percentage of non-carcinogens negative in the test
Predictivity	Percentage of all tested compound that was predicted correctly

The Ames assay

The Ames test is an assay that was developed by Bruce Ames and is performed to assess the mutagenic potential of chemical compounds [11,12]. The assay is performed in *Salmonella typhimurium* bacteria that carry mutations in genes involved in histidine biosynthesis. As a consequence the bacterial cells require histidine for growth and are so-called histidine auxotrophes. Mutagenic compounds can cause a reverse mutation which results in bacteria that can grow on a histidine-deficient medium. The number of bacteria that form colonies is then used as a measure for the mutagenic potential of a compound.

Several bacterial strains are used that have frameshift or point mutations in the genes required for histidine synthesis. These diverse strains are used to be able to detect mutagens acting via different mechanisms. Besides the mutations in the histidine synthesizing genes, the tester strains also have additional mutations to make the strains more sensitive for the detection of mutations. A mutation in the genes used for lipopolysaccharide synthesis makes the cell wall of the *Salmonella typhimurium* bacteria more permeable. Moreover, the strains have a mutation in their excision repair system [11].

The specificity of the Ames assay is relatively high in comparison to the other in vitro genotoxicity tests (Table 3). The sensitivity, specificity and predictivity of the Ames assay calculated by Kirkland et al. was 58.8%, 73.9%, and 62.5%, respectively [13].

To mimic metabolism in bacterial (and mammalian) mutagenicity assays, a liver fraction (S9 mixture) containing phase I and II drug metabolizing enzymes from Aroclor 1254 treated male Sprague-Dawley rats is used. Aroclor 1254 stimulates the AhR, pregnane X receptor (PXR) and CAR and leads to high levels of cytochrome P450 (CYP) 1A1, CYP1A2, CYP2B and CYP3A, which are involved in the activation of a large number of proximate genotoxicants. Assays are performed in the presence and absence of S9 mixture to study whether compounds are activated or inactivated by metabolism.

Table 3. The sensitivity, specificity and predictivity of the assays of the standard regulatory test battery for the assessment of genotoxic potential [13,14].

Assay	Sensitivity (%)	Specificity (%)	Predictivity (%)
Ames	58.8	73.9	62.5
Chromosome aberration (CA)	65.6	44.9	59.8
Mouse lymphoma TK (MLA)	73.1	39.0	62.9
Micronucleus in vitro	78.7	30.8	67.8
Micronucleus in vivo	40.0	75.0	48.0

The chromosome aberration assay

The chromosome aberration assay (CA) is performed in vitro in cultured mammalian cells. Structural and numerical damage is scored by microscopic examination of chromosomes in mitotic metaphase cells. Tests are carried out with and without S9

mixture [15,16]. This assay is often performed in Chinese hamster ovary k1 (CHO-k1) or lung cells (V79) or human lymphocytes. Scoring needs specialized training and experience.

The sensitivity and predictivity of this test are 65.6%, and 59.8% respectively. The specificity of this test is low with only 44.9% [13].

The mouse lymphoma TK assay

Thymidine monophosphate (TMP) is one of the four desoxyribonucleotide monophosphates, TMP does not undergo significant conversion to other nucleotides. This conservation makes the TMP pool size quite small and constant under normal growth condition. Therefore the TMP pool serves as a regulator for DNA synthesis. If TMP is replaced by a lethal TMP analogue, cells will die. The phosphorylation of these analogues is mediated by the "salvage" enzyme thymidine kinase (TK), which phosphorylates thymidine into TMP in mammalian cells. TK-deficient cells lack this enzyme activity and therefore are resistant to the cytotoxic effect of the lethal analogue. In the mouse lymphoma TK assay, the TK-competent L5178Y (TK+/+ or TK+/-) cells are treated with the test agents. After treatment, the cells are shifted to a selective medium containing a lethal TMP analogue such as trifluorothymidine (TFT). Normally most cells will die, however in the presence of a mutagenic compound, TK -/- cells might have been formed which are resistant to the cytotoxicity. The number of cell colonies on test plates is therefore a measure for genotoxicity. The size of the colonies gives information about about chromosome damage as large changes in the DNA inhibit growth and result in small colonies, whereas large colonies denote gene mutation.

The sensitivity and predictivity of the mouse lymphoma TK assay (MLA) are 73.1% and 62.9%. Similar to the chromosome aberration assay, the specificity of this assay is low with only 39.0% [13].

The micronucleus assay

The fourth regulatory genotoxicity assay is the micronucleus assay. Chromosomal fragments or complete chromosomes that are the result from DNA damage or errors in the separation of chromosomes during the cell cycle, can sometimes be found outside the nucleus in one of the daughter cells. After division of the nucleus these DNA fragments will decondensate and form a so called micronucleus. By using DNA staining techniques these micronuclei become visible and countable under the microscope. The number of these micronuclei per 1,000 (bi-nucleated) cells is used as a measure for genotoxicity. This assay can be performed in vitro on cell lines like CHO-k1. Micronuclei can also be measured in red blood cells and bone marrow obtained from in vivo experiments.

By using centromeric probes it is possible to determine whether micronuclei contain complete chromosomes or fragments of chromosomes. These results can then be used to

determine whether compounds have a clastogenic or aneugenic mode of action [17].

The sensitivity, specificity and predictivity of the in vitro micronucleus assay are 78.7%, 30.8%, and 67.8%, respectively. The specifivity of the in vivo micronucleus assay in bone marrow is much higher with 75%. The sensitivity of the in vivo test is lower with 40% and the predictivity is 48% [13, 14].

The impact of positive findings for genotoxic potential and follow up testing strategies

A decision tree for the tiered approach in regulatory genotoxicity testing is shown in Figure 2. In general, a combination of the Ames + MLA + (or) CA is used in regulatory in vitro testing for genotoxic potential [13]. A combination of the Ames + MLA + CA has a high sensitivity (84.7%) but low specificity (22.9%) for carcinogenicity [13]. When these tests show no genotoxic potential, the in vivo micronucleus test is performed. This in vivo test is performed as there are several compounds that are poorly detected in vitro. For example proximate carcinogens that are activated by phase II enzymes [18, 19]. When the in vivo micronucleus assay also shows a negative result it is likely that the compound has no genotoxic potential and the compound can proceed in development.

More research is needed in the rare situation, where the in vivo micronucleus assay gives a positive result after negative results in vitro. It has been shown that compounds that increase or decrease the core body temperature for a sustained period, compounds that increase the erythropoiesis in the bone morrow, and compounds that inhibit protein synthesis induce the number of micronuclei in bone marrow in vivo. Experiments to show these modes of action have been described by an IWGT working group [20]. Such positive results are mostly irrelevant for humans. Mechanistic data to demonstrate lack of clinical relevance for humans or a non-DNA reactive mechanism can lead to continuation of the further development. For non-DNA reactive genotoxicants (e.g. topoisomerase inhibitors and spindle poisons) a threshold might be justified. In case of a DNA reactive mode of action development is terminated.

In the case of a positive result in the in vitro genotoxicity assays it is required to perform at least two follow up in vivo genotoxicity tests. These are the in vivo micronucleus assay and another test. In the past the UDS tests was often used. But nowadays the Comet assay is more preferred in the testing for human pharmaceuticals [21]. This because it has been shown that most in vivo micronucleus negative carcinogens giving DNA adducts are detected in the Comet assay. Of these compounds <20% are detected in the UDS assay [21]. Besides these two in vivo assays it might be useful to perform assays to elucidate the mode of action causing the positive result.

Mostly the in vivo genotoxicity assays will show a negative result after a positive in vitro result as the in vitro assays give a high number of false positive results. In retrospective analysis Kirkland et al. showed that the genotoxicity battery used in the

tiered approach is highly sensitive. About 80%-90% of the carcinogens are detected, however, the specificity of especially the in vitro mammalian genotoxicity assays is very low [13, 22]. This is also supported by the retrospective analysis performed by Snyder and Green [23]. They showed that 50% of non-carcinogenic marketed drugs have a positive result in the mammalian genotoxicity assays, indicating the high false positive rate of these tests.

Two negative results in vivo overrule in principle a positive result in vitro, however in the case of development of pharmaceuticals for human use often additional investigations are performed to get a clue about the reason for the positive result. In the case, the in vivo tests are positive additional investigations might also be useful to show whether the positive in vivo result is relevant for humans or that the compound acts by a threshold mode of action. In this way the compound can be saved from attrition. A summary of human non-relevant, indirect or threshold mechanisms of genotoxicity is given in Table 4.

Table 4. Summary of human non-relevant, indirect or threshold mechanism of genotoxicity. The in vitro systems affected and the probability to obtain experimental evidence to support the mechanism are shown [24].

Mode of action	Description	In vitro system affected	Possibility to obtain experimental evidence
In vitro specific	Rat S9 mixture specific effects	All, except primary hepatocytes	Reasonable
	Feeding effects	Bacteria	Reasonable
Direct DNA effect but with a threshold	Azo- and nitro- reduction	Bacteria	Reasonable
	DNA repair deficiency	All	Difficult
	Inadequate detoxification	All	Reasonable
	Metabolic overload (production of reactive oxygen species, lipid peroxidation and sulphydryl depletion)	Mammalian cells	Reasonable
Indirect effect	Inhibition of topoisomerases	Mammalian cells	Reasonable
	Inhibition of kinases	Mammalian cells	Reasonable
	Inhibition of DNA polymerases	Mammalian cells	Reasonable
	Imbalance of DNA precursors	Mammalian cells	Reasonable
	Energy depletion	Mammalian cells	Difficult
	Inhibition of protein synthesis	Mammalian cells	Difficult
	Nuclease release from lysosomes	Mammalian cells	Difficult
	Protein denaturation		Difficult
	Aneuploidy		Reasonable
	High toxicity		Reasonable

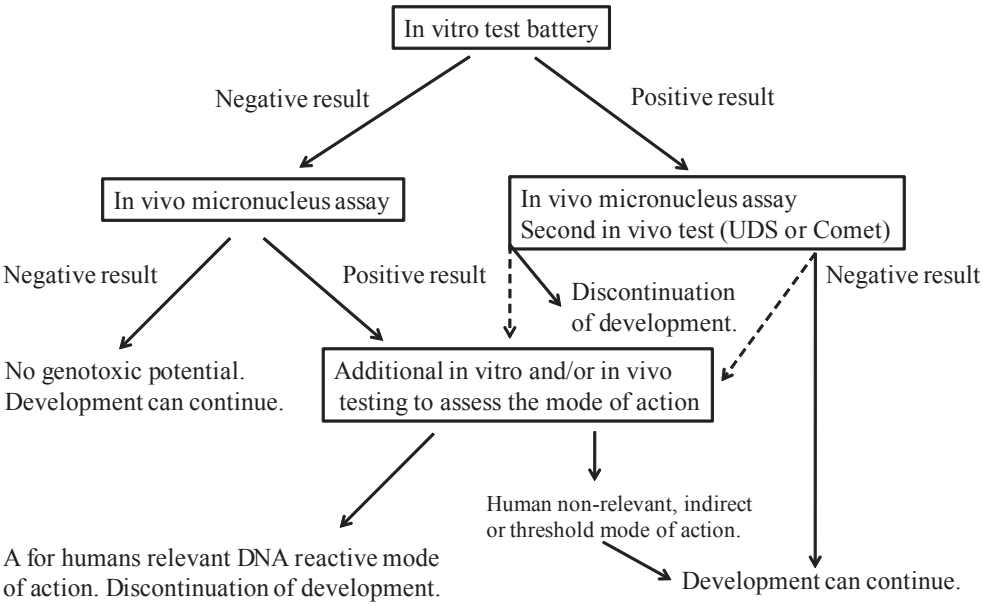

Figure 2. Schematic overview of the decision tree and necessary follow up testing for regulatory genotoxicity testing in case of pharmaceuticals for human application.

In a paper from Kirkland et al. [24], in vitro approaches are described to determine whether these effects occur in or are relevant for humans. The difficulty however is to predict what mechanism is affected by a compound giving a positive in vitro or in vivo genotoxicity result. Toxicogenomics approaches might be very valuable in this aspect as they can give a clue about the mechanism of action [25].

In general the following test strategy is used to assess the mode of action after a positive result in vitro that is suspected to be not relevant for the human situation or suspected to have a threshold [24]. Firstly in vitro assays are performed to show the indirect or threshold mode of action. Then in vivo tests are performed. When these tests are positive evidence must be obtained that this positive result is caused by the same mode of action. In case of a for human relevant non-DNA or threshold mode of action, the NOAEL (No Observed Adverse Effect Level) must be determined. When the anticipated human dose is much lower development of the compound might continue. In case of a for human relevant DNA reactive mode of action development of the compound is terminated.

Carcinogenicity testing

Testing of carcinogenicity is performed later in the process of drug development (parallel to phase II-III clinical studies) and is required for the final market approval of a compound

[26]. Upon availability of the results from the carcinogenicity studies, the genotoxicity results are used as part of the weight of evidence in cancer risk assessment.

The assessment of carcinogenicity is described in guideline ICH S1B for registration of pharmaceuticals for human use. According to this guideline the induction of tumors is monitored in a two-year, lifetime exposure of mice and rats [27]. As an alternative to the mouse bioassay a medium-term transgenic mouse model can be used [28]. There are several compounds that are carcinogens in rodents but act by a mechanism that is irrelevant for humans. For example compounds that activate the PPARα receptor in rodents. Therefore follow up testing to show the mechanism of action is necessary to assess relevance for humans.

Screening for compounds with genotoxic and carcinogenic potential in the discovery phase of drug development

The early screening of compounds for genotoxic/carcinogenic potential can be an attractive tool and makes use of these assays for the deselection and optimization of compounds in the discovery phase of drug development. This approach might be a way to improve the success rate of new chemical entities [29].

It has been shown that optimization of compounds in the early lead optimization phase on pharmacological properties as well as on pharmacokinetics/bioavailability is a successful strategy, that results in a lower drug attrition rate due to these properties [30]. Likewise this strategy might be useful in the case of genotoxicity/carcinogenicity.

The pharmaceutical industry has already put effort in the optimization of compounds to avoid attrition due to genotoxicity and carcinogenicity. In silico QSAR analysis is often applicated in the lead optimization phase. Software programs like DEREK, Topcat, Multicase, and Mutalert are routinely used during lead optimization [31]. In addition potential drugs are often tested in non-GLP versions of the micronucleus assay and miniaturized version of the Ames test at the end of the lead optimization phase just before the selection of the development candidates. These miniaturized versions of the Ames assay are still laborious and need approximately 300 mg of compound which makes application of the assay in the early lead optimization phase difficult [32].

Screening in the early lead optimization phase raises several issues. The earlier screening is performed in the process of drug development, the higher is the number of compounds that have to be tested. Furthermore, in the early phase of drug development only a small amount of compound is available. Early testing for toxicological hazard means also that the specificity of the assays has to be high as otherwise too many pharmacologically interesting compounds will be deselected. Therefore assays with a high-throughput and

specificity needing a low amount of compound are necessary. The exact number of compounds that have to be tested depends on the time point in the lead optimization phase where the assays are performed.

When assays are performed early in the lead optimization phase it is possible to use the assays for the optimization of compounds. When assays are used just before the selection of a development candidate, the assays are more deselection assays.

The regulatory genotoxicity assays have in general a very low throughput, need a high amount of compound, and are laborious. Therefore these assays are at least in their currently used format not suitable for medium- or high-throughput screening in the early discovery phase. Moreover, the specificity of most in vitro genotoxicity assays is too low to use them in the lead optimization phase. There are already a few commercially available screening assays for genotoxicity. However, these assays have in general been validated with only a limited number of compounds.

Thus in conclusion for early genotoxicity screening better in vitro assays with a higher throughput, a lower amount of compound required and a higher specificity are needed. These in vitro assays need validation with proper reference compounds.

Besides the detection of compounds that have the potential to be genotoxic carcinogens, it would be useful to detect compounds with non-genotoxic carcinogenic potential. As non-genotoxic carcinogens can have a wide variety of different mechanisms of action, this is even more challenging than testing for genotoxic potential. However, the activation of several receptors like the AhR, PPARα and CAR has been linked to non-genotoxic carcinogens. In these cases it might be possible to develop quick prescreens that measure the activation of such receptors.

The strategies that will be used in this thesis to develop, optimize and validate assays for genotoxic and non-genotoxic carcinogenicity are described on the next pages. High-throughput assays based on bacteria, yeast and human/rodent cell lines are proposed to be useful in vitro models. In the case of human cell lines, the focus is on the HepG2 cell line as, for reasons also outlined below, the properties of HepG2 cells are expected to give a good prediction for in vivo genotoxicity.

The detection of genotoxicity with bacterial screens

As an alternative for the Ames assay, the induction of genes involved in repair of bacterial DNA damage (SOS response) might be used as measure for the mutagenic potential of compounds. Assays that are based on these principles are the SOS chromotest and Vitotox™ assay.

The SOS chromotest is a colorimetric assay that measures the activation of the sfiA gene which is involved in the SOS response in bacteria [33]. In this assay, the promoter

of sfiA is linked to the lacZ gene. Activation of the SOS response therefore results in an increased production of the lacZ protein which can be measured colorimetrically. The Vitotox™ assay is a more modern version of this assay. The Vitotox™ assay is a bioluminescent assay with a medium-throughput test potential that also exploits the bacterial SOS response mechanism involved in DNA repair [34, 35]. One of the important proteins involved in the SOS response is the recN protein which is under non-stress conditions repressed by the LexA protein. In the Vitotox™ assay a genetically modified *Salmonella typhimurium* strain TA104 is used that contains the lux operon of *Vibrio fischeri* under transcriptional control of the promoter of the recN gene. After incubation of the bacteria in the presence of a genotoxic compound, the recN promoter is activated, as the repressor LexA protein is inactivated. This results in light production reflecting genotoxicity (bacterial mutagenicity). The applicability of this assay for screening in the discovery phase will be tested in Chapter 2 of this thesis.

The detection of genotoxicity with yeast screens

Bacterial tests have the disadvantage that these tests will not detect compounds that have solely a clastogenic or aneugenic mode of action. Eukaryotic yeast-based genotoxicity systems might be useful in this aspect. Like in the bacterial screens, the activation of genes involved in DNA repair might be used as a measure for detecting genotoxic potential in the assays. Several publications have described the application of a RAD54 promoter linked to a Green Fluorescent Protein (GFP) protein [36-40]. Induction of the RAD54 promoter due to DNA damage results in increasingly fluorescent cells. A quite similar assay is the RadarScreen assay which uses a RAD54 promoter linked to a β-galactosidase reporter that can be quantified by a luminometric measurement. This luminometric read-out will have the advantage that autofluorescence of compounds and S9 mixture does not hamper measurement. Studies to validate the RadarScreen assay with and without a S9 mixture are described in Chapter 2 of this thesis.

The detection of genotoxicity with human based cell line screens

Cell lines can also be used to determine the genotoxic potential of compounds. Human cell lines might even be more valuable as assays in these cells can reflect the human situation better than assays using bacteria, yeast and rodent cell lines. An important aspect for the development of a human based genotoxicity assays, is the choice of the primary and/or permanent cell line. At first instance primary cells seem the best choice.

However, for the fixation of mutations, induction of cell cycle checkpoints and DNA repair, cells need to divide. Most primary cells brought into culture are not yet adapted to the high oxygen concentrations in the cell incubators, therefore have a lot of stress and divide only a few times. Thus dividing permanent cell lines may be more preferable.

However, the problem with cell lines is that most have lost their p53 response, DNA repair response and metabolizing capacities. The application of the human hepatoma cell line HepG2 might be useful in this aspect because, as outlined in further detail below, these cells may display active p53, DNA repair and phase I and II metabolism. Three types of assays/techniques are applied in combination with the HepG2 cell line in this thesis. These are high content screening (HCS) to develop an in vitro micronucleus assay, toxicogenomics to find biomarkers for genotoxicity, and luciferase based reporter assays for the detection of genotoxic potential.

The HepG2 cell line

The HepG2 cell line is a liver hepatoma cell line that has retained activities of phase I and II metabolizing enzymes that are lost in most cultivated cells [41-43]. Normally S9 mixture is used to mimic metabolism in in vitro systems for genotoxicity testing. Although this mixture properly performs phase I metabolism, the metabolites might not be able to pass the human cell membranes due to the hydrophilicity of the metabolites. Other disadvantages of rat-S9 mixture are the absence of phase II metabolism and the fact that human specific metabolites will not be generated.

At our laboratory HepG2 cells were used in two cytotoxicity studies [44, 45]. Several compounds like for example benzo[a]pyrene of which the metabolites are known to be toxic, were toxic in this cell line. This implicated that phase I metabolic activity was present in these HepG2 cells. Besides phase I metabolism, phase II metabolism is also important for genotoxicity testing. Phase II enzymes can play a role in detoxification of reactive intermediates thereby reducing genotoxicity, but they may also play a role in bioactivation of proximate carcinogens. Phase II enzymes like sulfotransferases (SULTs) and N-acetyltransferases (NATs), for example, are important for the activation of many genotoxicants. Glatt et al. [18, 46] reported that more than 100 proximate mutagens are activated by SULTs. These SULTs are inactive in S9 mixture due to the lack of the proper cofactor in S9 mixture. Addition of the particular cofactor is not directly solving this problem since the conjugates cannot easily enter the cells due to their charge and hydrophilic character. Therefore SULT activated proximate genotoxicants are missed in the standard in vitro test systems, whereas they have been reported as genotoxic in HepG2 cells [47]. NAT activity is important for the detection of heterocyclic amines like 2-amino-3-methyl imidazo[4,5-f]quinoline (IQ) because such compounds require acetylation for their activation. The levels of these enzymes are low in CHO-k1 cells. As a consequence of NAT activity in HepG2 cells, results from in vitro micronucleus tests

with heterocyclic amines in HepG2 cells correlated much better with the carcinogenic effects in vivo than results obtained with other cell lines like CHO-k1 cells. Results from micronucleus tests in these HepG2 studies correlated even better with carcinogenicity in laboratory animals, than in vivo micronucleus tests in bone marrow of mice [48]. Further examples are the application of HepG2 cells to elucidate the genotoxic potential of mycotoxins [49, 50]. These compounds are carcinogens in vivo, genotoxic in HepG2 cells, but negative in all in vitro regulatory genotoxicity assays. Furthermore, HepG2 cells give promising results as several human specific carcinogens are detected. In summary, there are several studies that report bioactivation of proximate genotoxins/carcinogens by phase I or II metabolizing enzymes in the HepG2 cell line. However, a more detailed metabolic characterization of the HepG2 cell line is needed as it is not exactly known which phase I and II enzymes are present and inducible in the HepG2 cell line. This characterization is performed in Chapter 3 and 4 of the present thesis.

Another characteristic of the HepG2 cell line which is an advantage for genotoxicity testing is the fact that the HepG2 cell line has a functionally active p53 protein [51]. The tumor suppressor p53 is a potent transcription factor which stimulates the expression of genes involved in growth arrest, apoptosis, and DNA repair. Additionally, p53 is able to suppress the expression of a different set of genes including those involved in stimulating cell division [52]. Under normal circumstances, p53 is kept under tight control through its partnership with MDM2, an ubiquitin E3 ligase which mediates the ubiquitylation and degradation of p53. In response to a range of different cellular stresses [53], of which DNA damage is the most important one, p53-MDM2 protein interaction is interrupted with the result that p53 degradation is blocked [54]. As a consequence p53 is able to accumulate rapidly following DNA damage. The activation of p53-regulated genes results in either cell-cycle arrest allowing DNA repair or in p53-dependent apoptosis or cellular senescence in order to avoid the propagation of genetically defective cells [52, 54]. One of the activated genes of p53 is the cyclin dependent kinase (Cdk) inhibitor protein p21. This protein binds to G1/S-Cdk and S-Cdk and thereby blocks entry into the S-phase.

A functionally active p53 protein is also needed for a proper functional nucleotide excision repair and double strand break repair. Wang et al. [55] demonstrated that p53 plays a prominent role in the damage recognition and assembly of the repair machinery during global genome repair. The recruitment of the xeroderma pigmentosa C gene (XPC) which plays a prominent role in nucleotide excision repair was p53 dependent. Chang et al. [56] showed in the alkaline comet assay that after the introduction of p53 in p53-null cells the excision activity was restored. Similar results were reported in a micro-array experiment [57]. The basal expression of XPC was relatively low in lymphoblasts that did not have a functionally active p53 protein. After the onset of DNA damage by radiation only minor changes in the expression of XPC occurred. In this study similar

effects were observed for the radiation gene 51 homolog C (RAD51C) gene which plays a prominent role in double strand break repair [57].

Another, important characteristic of the HepG2 cell line is the presence of the nuclear transcription factor E2-related factor 2 (Nrf2) system [58]. Studies have shown that activation of the transcription factor Nrf2 induces the transcription of phase II detoxificating enzymes, antioxidant enzymes and transporters genes that protect against (geno-) toxic compounds. Examples of induced genes are glutathione S-transferase, NAD(P)H:quinone oxidoreductase 1, heme oxygenase-1, sulfotransferases, UDP-glucuronosyltransferase and multidrug resistance-associated protein. The protective effect is based on the fact that reactive oxygen species and (geno-)toxic compounds are metabolized and excreted in a relative fast way. Under homeostatic conditions in a cell, Nrf2 is retained in the cytoplasm by the Keap1 protein. Due to oxidative stress, Nrf2 is released from Keap1 and can translocate to the nucleus. There it activates genes with an electrophile responsive element (also called anti-oxidant responsive element) in the promoter as the above mentioned phase II metabolizing enzymes, transporters (phase III enzymes), and anti-oxidant genes.

The presence of metabolism, a functionally active p53 protein, active DNA repair, and an active Nrf2 system in HepG2 cells might give this cell line an advantage in (geno-) toxicity testing over the more commonly used cell lines, like V79 and CHO-k1 lines, in which these systems are absent or less functional. Several studies showed that the HepG2 cell line gives good results (high sensitivity and specificity) in genotoxicity assays like the in vitro micronucleus (IVMN) test and Comet assay [59-65]. However, the compound sets used in these studies were small and almost only positive controls were used.

The presence of phase II enzymes in the context of their detoxifying properties and the presence of a functional p53 and Nrf2 system in HepG2 cells might result in a reduction of the number of falsely predicted positive results. HepG2 cells might also prove to be an adequate tool to study the role of these systems in genotoxicity. Assays that will be developed/performed with HepG2 cells are described in the next sections.

The development of a high content screening micronucleus assay in HepG2 cells

High content screening (HCS) is the usage of (fluorescent) microscopy coupled with automated image analysis. Normally the scoring of micronuclei in microscopic slides under the microscope is performed by trained operators. This is laborious and therefore the throughput of the micronucleus assay is very low. By using the technique of HCS the throughput of the assay becomes high, also because it uses 96-well plates. This makes this assay suited for screening purposes in the discovery phase. An evaluation of this technique was performed by Diaz et al. [66]. An advantage of the HCS technique is that also other parameters like for example cytotoxicity can be measured in the same cells.

This improves the interpretation of genotoxicity results.

As stated before the in vitro mammalian genotoxicity assays in CHO-k1 and V79 cells give in general too many falsely predicted positive results for genotoxicity. Knowing the importance of the defense mechanisms (i.e. p53, Nrf2, DNA repair, phase II metabolism) against genotoxicity, it is noteworthy to realize that differences in the extent of presence/absence of these mechanisms in cell lines likely will have great influence on the sensitivity/specificity of the in vitro cell lines in comparison to cells in vivo.

Besides the development of a HCS micronucleus assay in HepG2 cells it will therefore be very useful to compare the sensitivity and specificity of the HepG2 cells with the commonly used CHO-k1 cells that lack many of the defense mechanisms (Chapter 5 of this thesis). This might give more insight into the reasons for the high number of falsely predicted positive results in the CHO-k1 cell line.

The development of luminescent based reporter assays in the HepG2 cell line

Human based genotoxicity assays for screenings purposes are rare, however, Gentronix has develop the Greenscreen HC GADD45a-GFP assay [67]. This assay links the regulation of the human GADD45a, which is induced after DNA damage, to the production of GFP. A disadvantage of this model system is that it has GFP as reporter gene. We identified that many compounds (from the legacy of Organon) interfere with GFP measurement because of autofluoresence at the same wavelength as GFP [4]. In addition, the human GADD45a cell line lacks metabolism. Application of S9 mixture is possible in the assay but this results in a low-throughput assay [67] with low sensitivity. Thus better assays are needed.

Luminescent based reporter assays in HepG2 cell lines might be useful as model systems. Luminescent based reporter assays in HepG2 cells will be developed in this thesis. Toxicogenomics data will be used to select proper biomarkers (genes or responsive elements) for the detection of genotoxic compounds (Chapter 6 of this thesis). It is expected that useful promoters and responsive elements will have a role in stress responses like the p53 response, Nrf2 response, DNA repair, apoptosis, and cell cycle control. A selection of promoters/responsive elements will be used to generate luminescent based reporter assays, which then will be validated (Chapter 7 of this thesis).

Detection of (species specific) non-genotoxic carcinogenicity

Several receptors have a role in non-genotoxic carcinogenicity like for example the aryl hydrocarbon receptor (AhR), peroxisome proliferator activated receptor alpha (PPARα), constitutive androstane receptor (CAR), estrogen receptor, thyroid hormone receptor,

androgen receptor and progesterone receptor [8]. This thesis is only focused on species differences in AhR activation between rat and human.

The AhR was discovered as the receptor that binds the environmental contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) [68]. The AhR is a basic helixloop-helix protein belonging to the Per-ARNT-Sim (PAS) family [69] and is located in the cytoplasm in an inactive complex with Hsp90 and p23. Binding of a ligand to the AhR leads to activation of the receptor and subsequently in its translocation to the nucleus. In the nucleus AhR releases its partner Hsp90 and forms a heterodimer with the AhR nuclear translocator (ARNT) protein. Further activation of this heterodimer by phosphorylation and/or dephosphorylation is required for DNA binding [70, 71].

Significant activation of the AhR by TCDD results in toxic effects like wasting syndrome, thymic involution, endocrine disorders, and very important teratogenicity and (nongenotoxic) carcinogenicity. Activation of the AhR with dioxin results in an increased incidence of liver tumors in both rats and humans. Studies in AhR-null mice have been used to investigate the role of the AhR in mediating the toxic effects. The studies revealed that these mice were resistant to TCDD induced lesions, strongly suggesting that the toxic effects are mediated by the AhR [72]. Furthermore, skin tumors that appear after topical application of the AhR agonist benzo[a]pyrene (B[a]P) are not formed in AhR-null mice [73].

Important for this non-genotoxic carcinogenic effect might be the change in expression patterns of several factors that are involved in cellular growth and differentiation. A second effect contributing to carcinogenicity may be the impairment of the p53 response [74, 75]. A third factor of importance is the strong induction of CYP1A1 which is involved in the metabolic activation of many potent proximate carcinogens like for example B[a]P.

Tumor promoting effects are both present in humans and rats although species dependent differences have been described [43]. The reported data about species differences are limited and therefore species differences in AhR induction in rat and human will be studied in Chapter 8 of this thesis.

Aim and outline of this thesis

The aim of the thesis was to develop high-throughput in vitro assays for the detection of compounds with genotoxic and non-genotoxic carcinogenic potential, validate these assays with proper reference compounds, and to develop a strategy for application of these assays in the early discovery phase of drug development. Such high-throughput assays may be applied for compound deselection, prioritization and/or optimization in the early discovery phase of drug development. A main goal was to develop in vitro

genotoxicity assays reflecting in vivo genotoxicity more accurately than the current assays.

Chapter 2 describes the validation of the high-throughput Vitotox™ (bacterial screen) and RadarScreen (yeast screen) assays. The validation of these two assays by using 62 compounds that are described in a compound list published by the European Centre for the Validation of Alternative Methods (ECVAM) and an additional set of 192 compounds is presented.

Chapter 3 describes the characterization of phase I metabolism in HepG2 cells. HepG2 cells were expected to give a better correlation with in vivo genotoxicity than yeast and other rodent cell lines like CHO-k1 and V79, which are often used in the regulatory in vitro studies for genotoxicity. Metabolic characterization of the HepG2 cell line was needed as conflicting results were reported about their metabolic capacities. We characterized the HepG2 cell line thoroughly under our culture conditions. The levels of CYP1A1, 1A2, 2B6, 2C9, 2D6, 2E1 and 3A4, NAT1 and EPHX1 in HepG2 cells were compared with the levels in primary human hepatocytes. Furthermore, their expression and enzyme activities after activation of the xenobiotic receptors AhR, PXR, CAR were measured.

In **Chapter 4**, the focus is on the characterization of HepG2 phase II metabolism. The expression levels and enzyme activities of several UGTs, SULTs, GSTs, NAT1 and EPHX1 were studied and compared to the levels in primary human hepatocytes. Moreover, the expression and enzyme activities after activation of the xenobiotic receptors AhR, PXR, CAR were assessed.

Chapter 5 describes the development of a high-throughput micronucleus assay in HepG2 and CHO-k1 cells by using high content screening. These high content screening micronucleus assays in CHO-k1 and HepG2 cells were validated with 62 compounds from an ECVAM compound list and 16 additional genotoxic reference compounds. The sensitivity and specificity of HepG2 and CHO-k1 cells were compared.

In **Chapter 6** gene expression profiling in HepG2 cels was used to find genes and responsive elements that can assess the genotoxic potential of compounds and discriminate genotoxic carcinogens from non-genotoxic liver toxicants.

Based on the HepG2 toxicogenomics data and data from literature four mechanism-based HepG2 luciferase reporters assays were developed (**Chapter 7**). The promoter regions of RAD51C and cystatin A, as well as the responsive element of the p53 protein, were used. In addition, a luciferase based reporter assay was generated that measures the activation of the Nrf2 oxidative stress pathway. The assays were thoroughly validated with 62 reference compounds described by the ECVAM and an additional set of 192 compounds.

In **Chapter 8** the focus is on non-genotoxic carcinogenicity that is related to activation of the AhR receptor. Species differences between activation of the rat and human AhR

were studied.

Finally the thesis is concluded with a summary, discussion, conclusion, and additional considerations in **Chapter 9**.

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Chapter 2

Evaluation of the Vitotox™ and RadarScreen assays for the rapid assessment of genotoxicity in the early research phase of drug development

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Abstract

The Vitotox™ and RadarScreen assays were evaluated as early screens for bacterial mutagenicity and in vitro mammalian genotoxicity, respectively. The Vitotox™ assay is a bacterial reporter assay in *Salmonella typhimurium* based on the SOS response, and it contains a luciferase gene under control of the recN promoter. The RadarScreen assay is a RAD54 promoter-linked β-galactosidase reporter assay in yeast.

Recently an ECVAM workgroup defined a list of 20 genotoxic and 42 non-genotoxic compounds [Mutat. Res 653 (2008) 99-108], that can be used for the validation and/or optimization of in vitro genotoxicity assays. In the present study, this compound set was used for the validation of the assays. Moreover, an additional set of 192 compounds was used to broaden this validation study. The compounds of this additional set can be classified as non-genotoxins and genotoxins and consists of both in-house and reference compounds. In case of the ECVAM compound list, the results from the Vitotox™ and RadarScreen assays were compared to the genotoxic/non-genotoxic classification of the compounds in this list. In case of the additionally tested compounds, the results of the Vitotox™ and RadarScreen assays were compared, respectively, to bacterial mutagenicity (Ames) or in vitro mammalian genotoxicity data obtained in-house or from the literature.

The validation with respect to the ECVAM compound list resulted in a sensitivity for both the Vitotox™ and RadarScreen assay of 70% (14/20). If both assays were combined the sensitivity increased to 85% (17/20). Both tests also gave a low number of false positive results. The specificity of the Vitotox™ and RadarScreen assay was 93% (39/42) and 83% (35/42), respectively. This resulted in a predictivity of the Vitotox™ and RadarScreen assay of 85% (53/62) and 79% (49/62), respectively. In case both tests were combined the specificity and the predictivity of the Vitotox™ and RadarScreen assay appeared 81% (34/42) and 82% (51/62), respectively.

The results from the additional list of 192 compounds confirmed the results found with the ECVAM compound list. The results from the Vitotox™ assay showed a high correlation with Ames results of 91% (132/145). Subsequently, the RadarScreen assay had a correlation with in vitro mammalian genotoxicity of 76% (93/123). The specificity of the Vitotox™ assay was 94% (90/96) for Ames results and that of the RadarScreen assay was 74% (34/46) for in vitro mammalian genotoxicity. Moreover, the sensitivity of the Vitotox™ and RadarScreen assays were 86% (42/49) and 77% (59/77), respectively.

Implementation of the Vitotox™ and RadarScreen assays in the early research phase of drug development can lead to fast deselection for genotoxicity. It is expected that this application will reduce the number of compounds that have a positive score in the regulatory Ames and in vitro mammalian genotoxicity tests.

Introduction

Screening on genotoxicity and immediate deselection of genotoxic compounds in the early research phase of drug development can improve the success rate of new chemical entities. In the early research phase, medium- or high-throughput toxicity assays are a prerequisite as the number of compounds is high while the available amount of compound is limited.

The regulatory genotoxicity tests like the Ames, micronucleus (MN), chromosome aberration (CA), mouse lymphoma assay (MLA), and sister chromatid exchange (SCE) tests are all relatively time-consuming and laborious and therefore, at least in their present format less applicable as an early screen.

The Vitotox™ and RadarScreen are two simple assays that may be useful for early genotoxicity screening. The Vitotox™ assay is a bacterial reporter assay based on the SOS response system; it contains a luciferase gene under control of the recN promoter [1]. Normally, this recN promoter is strongly repressed, but in the presence of a DNA-damaging genotoxic compound, the RecA regulator protein recognizes the resultant free ends or mismatches in DNA. This initiates a cascade of reactions known as the SOS response that cleaves the LexA repressor and de-represses the strong recN promoter, which leads to an increased luciferase expression. This increase can be quantified by measurement of luminescence after addition of luciferin.

The results from the Vitotox™ assay correlate good with results from the Ames test [2]. Furthermore, for many compounds the lowest effective concentration (LEC) in the Vitotox™ assay was 5-100 times lower than the LEC measured in the Ames test [2]. This higher sensitivity for several compounds may be explained by the fact that induction of DNA repair already occurs at lower compound concentrations than fixation of a mutation. Furthermore, the Ames assay measures only one specific mutation per strain.

The principle of using the induction of the SOS response in bacteria as a measure for Ames positive results has been used in several assays. Several genes playing a role in the SOS response were used as reporter genes. The SOS chromotest which is one of the best known of these assays has been validated in several studies [3-5]. One of the largest validation studies was performed by Mersch-Sundermann et al. [3], who tested a set of 330 chemicals. The predictivity of the SOS chromotest for the Ames test was 86.4% in this study (sensitivity, 78.6%; specificity, 100%). These results showed that the induction of the SOS response might be a good predictor for Ames results. This was the reason that the Vitotox™ assay was evaluated as an early screen to detect Ames-positive compounds.

The RadarScreen assay is based on yeast strain SKAM4 that contains a RAD54 β-galactosidase reporter construct (reMYND, Leuven, Belgium). This β-galactosidase gene is under the transcriptional control of the RAD54 promoter and is expressed after

DNA damage to repair double-strand breaks by homologous recombination [6, 7]. Compounds supplied to the growth medium can therefore be evaluated for genotoxicity by determining their effect on reporter gene expression. The quantity of β-galactosidase can be measured easily by the addition of its specific substrate D-luciferin-o-β-galactopyranoside (Promega, Madison, USA), which is cleaved into galactose and luciferin. The latter product can be measured luminometrically. The advantage of yeast compared with bacteria, is that clastogenic and aneugenic compounds causing chromosome aberrations, micronuclei formation and improper chromatid exchange may also be detected. The RAD54 promoter is also used in the GreenScreen assay from Gentronix [8, 9]. This GreenScreen assay is quite similar to the RadarScreen assay, however, it has a different read-out system with a Green Fluorescent Protein (GFP) gene under control of the RAD54 promoter. The advantage of the RadarScreen over the use of the GreenScreen assay is the sensitive read-out of luminescence. This even makes the use of S9 mixture for metabolic activation in a medium- or high-throughput assay setup possible. Furthermore, many (pharmaceutical) compounds can cause autofluorescence and may interfere with the GFP measurement. Approximately 25% of the in-house compounds were missed due to these effects [10]. This can be prevented with a luminescent read-out.

A big concern with in vitro genotoxicity assays is the high number of (irrelevant) positive results compared with the in vivo results. For this reason the European Centre for the Validation of Alternative Methods (ECVAM) recently published a list of 62 compounds that can be used for the validation/optimization of (new) in vitro genotoxicity tests [11]. Based on the available data, the compounds were defined by an expert panel as genotoxic or non-genotoxic. The list of compounds was divided into 20 genotoxic and 42 non-genotoxic compounds. The latter group also contained 19 non-genotoxic compounds that often give false positive results in in vitro genotoxicity assays. In the present validation study the compounds of this ECVAM list were tested. Also an additional set of 192 compounds was tested to broaden the study in order to obtain a higher comfort level. The compounds in this additional set can be classified as non-genotoxins or genotoxins, and consisted of both in-house and reference compounds. The genotoxins acted via diverse mechanisms and belong to the direct-acting genotoxins (i.e. methyl methane sulfonate (MMS) and 4-nitroquinolin-1-oxide (4NQO), cross-linking agents (e.g. cisplatin), topoisomerase inhibitors (e.g. doxorubicin and ellipticin), nucleotide/DNA synthesis inhibitors (i.e. 5-fluorouracil and methotrexate), reactive oxygen species generators (i.e. hydrogen peroxide) and aneugens (i.e. colchicine). Furthermore the compound list contains several steroidal compounds which have been reported as being positive in in vitro mammalian genotoxicity tests [12].

In case of the ECVAM compound list the results from the Vitotox™ and RadarScreen assays were compared to the genotoxic/non-genotoxic classification of the compounds

in this list. In case of the additionally tested compounds, the results of the Vitotox™ assay were compared with Ames scores. Subsequently, the results from the RadarScreen assay were compared with in vitro mammalian genotoxicity data obtained in-house or from the literature. These two comparisons were performed as the Vitotox™ assay is a pre-screen for bacterial mutagenicity (Ames test) and the RadarScreen assay is a pre-screen for in vitro mammalian genotoxicity.

Materials and methods

Preparation of compound solutions

Stock solutions of the reference and test compounds were freshly prepared in 100% dimethyl sulfoxide (DMSO). From the stocks, √10-fold dilution series were prepared in DMSO. All compounds were of analytical grade and ordered from Sigma-Aldrich or synthesized in-house.

Vitotox™ assay

The Vitotox™ assay kit was purchased from Thermo Electron Corporation (Vantaa, Finland) and handled according to the manufacturer's manual. Bacteria from the recN2-4 strain, used for the induction of SOS repair, and the pr1 strain, used for cytotoxicity measurement, were grown overnight. Cultured bacteria were used when the optical-density measurements at 595 nm (OD595) of the recN2-4 and pr1 strains were between 0.2-0.5 and 0.4-0.6, respectively. When the densities were below these values the culture time was prolonged. After reaching the optimal densities, recN2-4 and pr1 cultures were diluted 10- and 2-fold with growth medium and water, respectively. For metabolic activation of the test compounds S9 mixture was added (V:V, 1:10) to this growth medium. S9 mixture consisted of 2.5% liver-S9 homogenate from Aroclor induced Wistar rats (NOTOX, 's-Hertogenbosch, The Netherlands), 15.2 mM KCl, 4.28 mM $MgCl_2$, 1.86 mM β-NADP, 9.29 mM glucose 6-phosphate, and 93 mM phosphate buffer pH 7.4. The assay was carried out in white 384-well plates (Perkin-Elmer, Groningen, The Netherlands). To the plates 9 µl of compound solution was added leading to a final concentration of DMSO in the well of 0.1 or 1%. Next 81 µl diluted recN2-4 or pr1 culture solution in the absence or presence of S9 mixture was added. Plates were sealed and the luminescence was read on the Victor II (Perkin-Elmer, Groningen, The Netherlands) at 26 ºC every 5 min for a period of 3 h.

After completion of the assay the signal-to-noise ratio (S/N) of each measurement was calculated, i.e. the luminescence of exposed bacteria divided by the luminescence of DMSO treated bacteria. Values measured during the first 45 min were not used since a

SOS response can not occur that quickly. A compound was considered to be genotoxic when the S/N ratio of the recN2-4 strain was greater than 1.3 and the S/N ratio of the recN2-4 strain divided by the S/N ratio of the pr1 strain was greater than 1.5. The highest test concentration in the Vitotox™ assay was limited at 0.1 mM, unless indicated otherwise.

RadarScreen assay

Yeast was grown overnight and thereafter the OD595 was measured. The yeast suspension was diluted with growth medium to an OD595 of 0.5. For testing with metabolic activation, the S9 mixture was prepared as described for the Vitotox™ assay, however, in case of the RadarScreen assay the final concentration S9-liver fraction was 2%. It is of practical importance that S9 mixture is mixed with the yeast suspension in advance of the addition to the wells. The assay was carried out in white and transparent 96-well plates (Perkin-Elmer). A volume of 10 µl compound solution was added leading to a final concentration of 1% DMSO in the assay. Subsequently, 90 µl of the yeast suspension in the presence or absence of S9 mixture was added. Plates were incubated for 6 h at 30 ºC. After the incubation period the absorbance was measured at 590 nm in the transparant plates with the Victor II. The decrease in the OD values of 1 mM MMS was set as 100% cytotoxicity, because almost all cells were killed at this concentration. The cytotoxicity of all other compounds was calculated relatively to MMS. In addition, blank plates containing yeast medium and only the compound concentrations similar to those in the test plates were checked for precipitation by visual inspection.

A volume of 50 µl Beta-Glo substrate (Promega, Madison, USA) was added to the plates. This β-galactosidase substrate is cleaved by β-galactosidase into galactose and luciferin. The luciferin is subsequently utilized by firefly luciferase to generate light. After addition of this substrate the plates were shaken for 45-60 minutes to allow cell lysis. Then the luminescence was measured on the Victor II. A compound was considered to be genotoxic when the response was induced by a factor 1.5 (50% increase=5.4 times the standard deviation of the background). Background means here yeast cells treated with 1% DMSO. In total 120 untreated controls were used to assess this value (derived from 10 independent experiments). The highest test concentration in this assay was 1 mM. This top dose is the same as the recommended top dose for the regulatory in vitro mammalian genotoxicity (clastogenicity) tests. Two aspects that can cause false positive results are cytotoxicity and precipitation. Therefore, it was decided to discard measurements showing precipitation or more than 80% cytotoxicity.

Calculations

Compounds that are positive in the Vitotox™ or RadarScreen assay can show a positive ('A') or negative results in a second test ('B'). Similarly, compounds with a negative score in the Vitotox™ or RadarScreen assay can give a positive ('C') or negative ('D') score in a second test. The second test that is used for a comparison gives A+C positive scores and B+D negative scores. By using these values the sensitivity, specificity and predictivity can be calculated: sensitivity = A/(A+C); specificity = D/(B+D); predictivity/correlation = (A+D)/(A+B+C+D).

In case of the ECVAM compound list, the results from the Vitotox™ and RadarScreen assay were compared to the (in vivo) genotoxic/non-genotoxic classification of the compounds. In case of the additional list of compounds the results of the Vitotox™ and RadarScreen assay were respectively compared to the available Ames or in vitro mammalian genotoxicity data from in-house or from literature. In case the results from the screening tests were inconsistent with the results from the Ames or in vitro mammalian genotoxicity tests these results were called 'false positive' or 'false negative'. A compound with one positive score in an in vitro mammalian genotoxicity assay was defined as positive (genotoxic). Compounds with inconsistent Ames data were discarded from the calculations.

Results

Validation of the assay procedure with reference compounds and reproducibility of the assay method

4-Nitroquinoline-1-oxide (4NQO) and benzo[a]pyrene (B[a]P) were used as positive controls in the Vitotox™ assay. The concentration-dependent response is shown in Figure 1. As expected, the direct-acting genotoxin 4NQO showed a concentration-related induction of the luminescence in the absence of S9 mixture. However, due to cytotoxicity the SOS response started later for 4NQO at a concentration of 10^{-4} M. In the presence of S9 mixture the genotoxic activity was decreased (detoxification). However, at higher concentrations activation of the SOS response was visible again. In the absence of S9 mixture B[a]P showed no enhancement of the luminescence. B[a]P was activated by the addition of S9 mixture and showed a concentration-dependent increase in the induction of the luminescence.

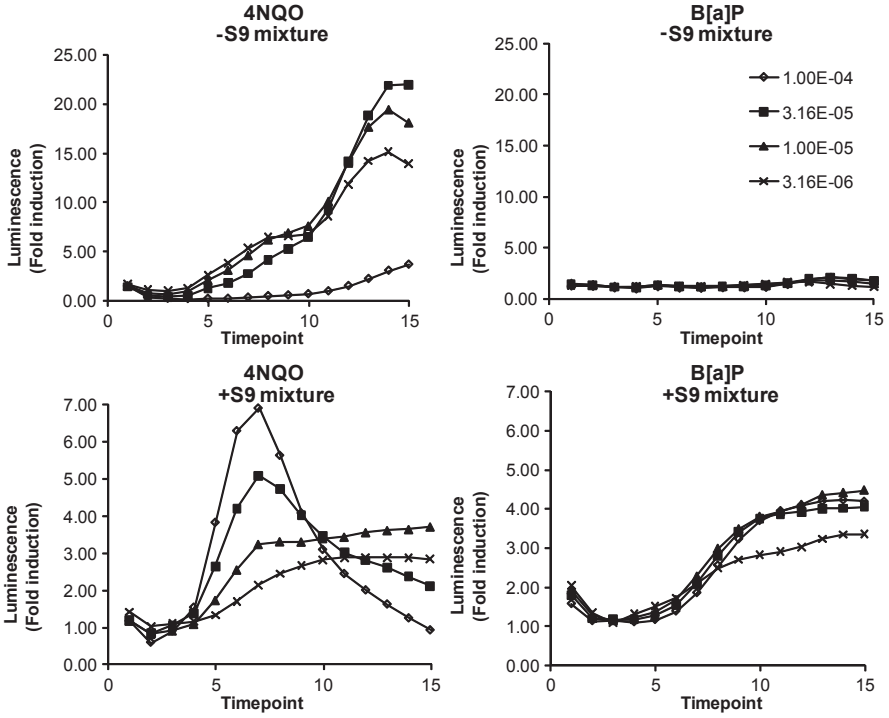

Figure 1. Concentration-dependent genotoxicity of 4-nitroquinoline-1-oxide (4NQO) and benzo[a] pyrene (B[a]P) in the Vitotox™ assay in the absence and presence of S9 mixture. Compounds were tested in 20 independently performed experiments. A representative curve is shown.

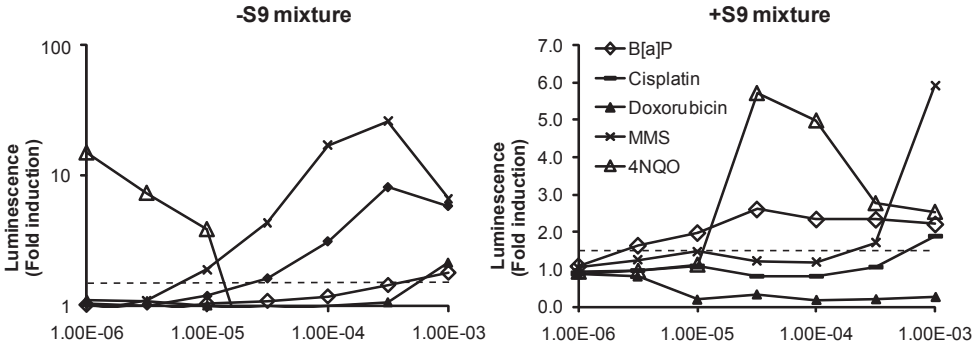

Figure 2. Concentration dependent genotoxicity of benzo[a]pyrene (B[a]P), cisplatin, doxorubicin, methyl methanesulfonate (MMS) and 4-nitroquinoline-1-oxide (4NQO) in the RadarScreen assay in the absence and presence of S9 mixture. Compounds were tested at least in two independently performed experiments. A representative curve is shown. The dashed line represents the genotoxicity threshold.

MMS and B[a]P were used as positive controls in the RadarScreen assay. The concentration-dependent response of these two positive controls and a representative set of 3 other genotoxins being cisplatin, doxorubicin and 4NQO are shown in Figure 2. The compounds were tested in at least two independent experiments. B[a]P already showed a genotoxic effect in the absence of S9 mixture. However, this effect was only seen at very high concentrations as the LEC was 10^{-3} M. At this concentration an induction of the luminescence of 1.8-fold was observed. As expected, the addition of S9 mixture resulted in metabolic activation of B[a]P. In the presence of S9 mixture the LEC was 3.16×10^{-6} M and a maximum induction of 2.6-fold was observed at a concentration of 3.16×10^{-5} M.

Cisplatin showed the largest activity in the absence of S9 mixture. The LEC of cisplatin was 3.16×10^{-5} M in the absence of S9 mixture and 1.00×10^{-3} M in the presence of S9 mixture. Doxorubicin only showed activity at the highest test dose of 1.00×10^{-3} M in the absence of S9 mixture. MMS showed activity in the presence and absence of S9 mixture, but the largest activity was shown in the absence of S9 mixture with a LEC of 10^{-5} M. Likewise, 4NQO showed the highest activity in the absence of S9 mixture. In absence of S9 mixture the LEC was $\leq 10^{-6}$ M and after the addition of S9 mixture the LEC increased to 3.16×10^{-5} M. At the highest test concentrations the luminescent signal showed a steep decrease due to the cytotoxic effect of 4NQO.

To show the reproducibility of the RadarScreen assay, MMS and B[a]P were tested in nine independent experiments (Figure 3). Without S9 mixture MMS had a LEC of 3.16×10^{-6} M in six experiments. In the remaining three experiments the LEC was 1.00×10^{-5} M. In the presence of S9 mixture, MMS was less genotoxic and the variation

in the LEC was larger. The LEC was $3.16x10^{-5}$ M in two experiments, $1.00x10^{-4}$ M in two experiments, $3.16x10^{-4}$ M in three experiments, and $1.00x10^{-3}$ M in two experiments. The results observed for B[a]P showed a high reproducibility. Without S9 mixture B[a]P had a LEC of $3.16x10^{-4}$ M in eight experiments. Only in one experiment the LEC was $1.00x10^{-3}$ M. In the presence of S9 mixture B[a]P was activated and the LEC was $3.16x10^{-6}$ M in seven experiments and $1.00x10^{-5}$ M in two experiments. The experiments with these two reference compounds give a first indication that the reproducibility of the RadarScreen assay is good.

The reproducibility of the cytotoxicity measurement in the RadarScreen was also tested. In nine independently performed experiments, the cytotoxicity of MMS was assessed in the absence and presence of S9 mixture. The high reproducibility of this method is shown in Figure 4. The highest test concentration of MMS kills almost all yeast cells and reduces the OD590 in all experiments almost to the background level (wells with culture medium and 1% DMSO). The toxicity of the other compounds was calculated relatively to the toxicity of $1.00x10^{-3}$ M MMS, which was set at a toxicity of 100%

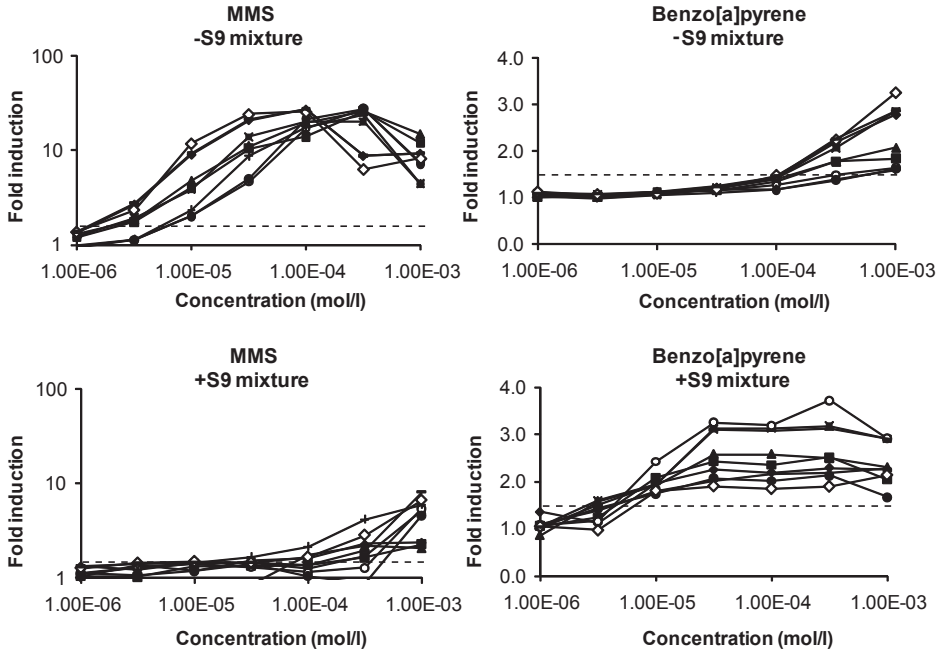

Figure 3. The reproducibility of the concentration-dependent genotoxicity of methyl methanesulfonate (MMS) and benzo[a]pyrene in the RadarScreen assay in the absence and presence of S9 mixture in nine independently performed experiments. The dashed line represents the genotoxicity threshold.

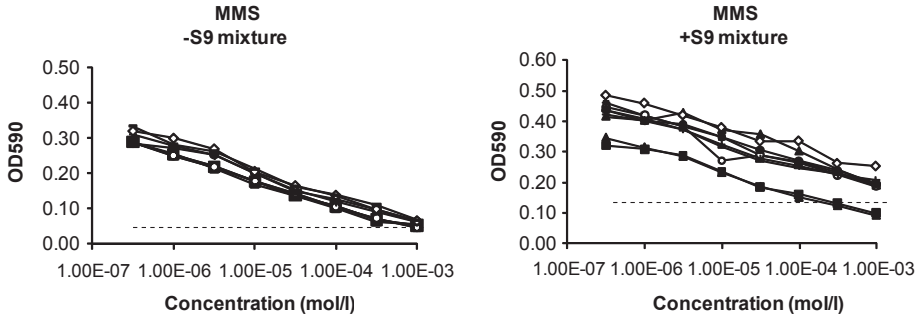

Figure 4. The reproducibility of the concentration dependent cytotoxicity of methyl methanesulfonate (MMS) in the RadarScreen assay in the absence and presence of S9 mixture in nine independently performed experiments. The dashed line represents the background. The lowest concentration in the curves represents the control (yeast + 1% DMSO).

Validation of the Vitotox™ and RadarScreen assays with respect to the ECVAM compound list

The effects of the 62 ECVAM compounds were tested in the Vitotox™ and RadarScreen assay. The overall results are shown in Tables 1-3.

The sensitivity, specificity and predictivity of the two single tests and the combination of these tests were calculated (Table 4). The sensitivity to detect genotoxic compounds was 70% (14/20) for both the Vitotox™ and RadarScreen assay. When results from both tests were combined the sensitivity increased to 85% (17/20). Thereby the tests detect a different but overlapping set of compounds. Sodium arsenite, taxol and chloramphenicol were not detected with both assays (Table 1).

Both tests have a high specificity of more than 83% which means that the number of false positives in both tests is low. The number of false positive results slightly increased after combining the tests, however, the specificity still remains high with 81% (34/42). Of the 19 compounds that give often false positive results only four (tertiary-butylhydroquinone, curcumin, sodium xylene sulfonate and 2,4-dichlorophenol) and two (tertiary-butylhydroquinone and 2,4-dichlorophenol) compounds gave a positive score in the RadarScreen and Vitotox™ assays, respectively. Of the non-DNA reactive chemicals that have not been reported to give positive results in vitro (23 compounds), ampicillin trihydrate, progesterone and hexachloroethane gave a positive result in the RadarScreen assay. In the Vitotox™ assay only phenanthrene gave a positive result. The overall predictivity of both tests for genotoxicity is 82% (51/62), while these values were 85% (53/62) and 79% (49/62) for the Vitotox™ and RadarScreen assay, respectively.

Table 1. Results from the Vitotox™ and RadarScreen assays regarding the ECVAM compounds that were defined as in vivo genotoxins that should be detected as positive in in vitro mammalian cell genotoxicity tests. The false negative results are indicated in bold.

Chemical	CAS number		Vitotox™	LEC (mol/l)	RadarScreen	LEC (mol/l)
I. Ames-positive in vivo genotoxins						
(i) O^6 and N^7 alkylators						
Cyclophosphamide	6055-19-2	Requires metabolic activation (CYP2B6)	**N**		Y(S9)[a]	$3.16x10^{-4}$
ENU	759-73-9	Strong gene mutagen (O^6 alkylation)	Y	$3.16x10^{-5}$	Y(S9)[a]	$3.16x10^{-5}$
MMS	66-27-3	Strong clastogen (N^7 alkylation)	Y	$1.00x10^{-5}$	Y	$3.16x10^{-6}$
(ii) Polycyclic aromatic hydrocarbons						
Benzo[a]pyrene	50-32-8	Requires metabolic activation (CYP 1A1; 1B1, epoxide hydrolase); forms bulky adducts	Y(S9)	$3.16x10^{-6}$	Y(S9)[a]	$1.00x10^{-5}$
7,12-Dimethylbenzanthracene	57-97-6	Requires metabolic activation (CYP1B1); forms bulky adducts	Y	$1.00x10^{-5}$	Y	$3.16x10^{-4}$
(iii) Aromatic amines						
Dimethylnitrosamine	62-75-9	Alkylating agent after activation by CYP2E1(which is not highly expressed in rat liver S9): produces O^6- and N^7-methyl guanine adducts	**N**		Y(S9)	$1.00x10^{-4}$
2-Acetylaminofluorene	53-96-3	Hydroxylated by CYP1A2 and then acetylated. Forms C8 adduct on guanine	Y(S9)	$1.00x10^{-4}$	Y(S9)	$1.00x10^{-4}$
2,4-Diaminotoluene	95-80-7	Aromatic amine, requires metabolic activation	Y(S9)	$1.00x10^{-4}$	Y(S9)	$3.16x10^{-6}$
IQ (2-amino-3methylimidazo[4,5-*f*] quinoline)	76180-96-6	Heterocyclic amine with potent genotoxicity, requires metabolic activation	Y(S9)	$3.16x10^{-6}$	Y(S9)	$3.16x10^{-5}$
PhIP.HCl (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine		Heterocyclic amine with potent genotoxicity, requires metabolic activation	Y	$3.16x10^{-6}$	**N**	
(iv) Others						
Aflatoxin B1	1162-65-8	Activated by CYP3A4, which is not highly expressed in rats compared with humans. Forms various adducts	Y	$3.16x10^{-6}$	Y(S9)	$3.16x10^{-5}$
Cadmium chloride	10108-64-2	Inorganic carcinogen	**N**		Y	$3.16x10^{-6}$
Cisplatin	15663-27-1	Cross-linking agent	Y	$3.16x10^{-6}$	Y	$3.16x10^{-5}$
p-Chloroaniline	106-47-8	No adducts	Y(S9)[a]	$3.16x10^{-5}$	Y(S9)	$1.00x10^{-3}$
II. In vivo genotoxins negative or equivocal in Ames						
Etoposide	33419-42-0	Topoisomerase inhibitor	Y(S9)	$3.16x10^{-5}$	Y(S9)	$3.16x10^{-5}$
Hydroquinone	123-31-9	MOA: aneugen	Y(S9)	$1.00x10^{-4}$	**N**	
Azidothymidine	30516-87-1	MOA: nucleoside analogue	Y	$3.16x10^{-6}$	**N**	
Sodium arsenite	7784-46-5	Inorganic carcinogen MOA: oxidant? repair inhibitor?	**N**		**N**	
Taxol	33069-62-4	MOA: aneugen	**N**		**N**	
Chloramphenicol	56-75-7	MOA: clastogen that binds to DNA	**N**		**N**	

[a] Compounds that are activated by S9 mixture but already show some activity without the addition of S9 mixture.

Table 2. Results from the Vitotox™ and RadarScreen assays regarding the ECVAM compounds that were defined as non-DNA-reactive chemicals (including non-genotoxic carcinogens), metabolic poisons and others that should give negative results in in vitro genotoxicity tests but have been reported to induce chromosomal aberrations or tk mutations in mouse lymphoma cells, often at high concentrations or at high levels of cytotoxicity. The false positive results are indicated in bold.

Chemical	CAS number	Vitotox™	LEC (mol/l)	Radar-Screen	LEC (mol/l)
I. Non-carcinogens that are negative or equivocal for genotoxicity in vivo					
D,L-menthol	15356-70-4	N		N	
Phthalic anhydride	85-44-9	N		N	
Tertiary-butylhydroquinone	1948-33-0	**Y(S9)**	$3.16x10^{-6}$	**Y(S9)**[a]	$3.16x10^{-6}$
o-Anthranilic acid	118-92-3	N		N	
1,3-Dihydroxybenzene (resorcinol)	108-46-3	N		N	
2-Ethyl-1,3-hexanediol	94-96-2	N		N	
Sulfisoxazole	127-69-5	N		N	
II. Non-carcinogens with no in vivo genotoxicity data					
Ethionamide	536-33-4	N		N	
Curcumin	458-37-7	N		**Y**	$1.00x10^{-5}$
Benzyl alcohol	100-51-6	N		N	
Urea	57-13-6	N		N	
II. Non-genotoxic carcinogens or carcinogenic by irrelevant (for humans) mechanism					
Sodium saccharin	128-44-9	N		N	
IV. Supplementary list (prediction of in vitro genotoxicity results less clear)					
Propyl gallate	121-79-9	N		N	
p-Nitrophenol	100-02-7	N		N	
Sodium xylene sulfonate	1300-72-7	N		**Y**	$1.00x10^{-3}$
Ethyl acrylate	140-88-5	N		N	
Eugenol	97-53-0	N		N	
Isobutyraldehyde	78-84-2	N		N	
2,4-Dichlorophenol	120-83-2	**Y**	$1.00x10^{-4}$	**Y**	$3.16x10^{-4}$

[a] Compounds that are activated by S9 mixture but already show some activity without the addition of S9 mixture.

Validation of the Vitotox™ and RadarScreen assays with respect to the additional set of 192 compounds

Besides the compounds from the ECVAM list, the assays were further validated with an additional set of 192 compounds. The data set was not complete for all the compounds. Ames data were available for 145 compounds and in vitro mammalian genotoxicity data for 124 compounds. The results from the Vitotox™ and RadarScreen assays and additional genotoxicity data that were available for these compounds are shown in Tables 5 and 6. From the in-house prepared compounds the Org codes and chemical names are summarized in Table 7.

As the Vitotox™ assay is meant as a pre-screen for the Ames assay, the results from this test were compared to Ames results. The sensitivity, specificity and predictivity/correlation of the Vitotox™ results for Ames scores were calculated (Table 8). The RadarScreen is a pre-screen for in vitro mammalian genotoxicity (chromosome damage). Therefore the sensitivity, specificity and predictivity/correlation of the RadarScreen results for in vitro mammalian genotoxicity scores were calculated (Table 8).

Table 3. Results from the Vitotox™ and RadarScreen assays regarding the ECVAM compounds that were defined as non-DNA-reactive chemicals (including non-genotoxic carcinogens).The false-positive results are indicated in bold.

Chemical	CAS number	Vitotox™	LEC (mol/l)	Radar Screen	LEC (mol/l)
I. Non-carcinogens with negative in vivo genotoxicity data					
Ampicillin trihydrate	7177-48-2	N		**Y(S9)**	$1.00x10^{-3}$
D-mannitol	69-65-8	N		N	
II. Non-carcinogens with no in vivo genotoxicity data					
Phenformin HCl	834-28-6	N		N	
n-Butyl chloride	109-69-3	N		N	
(2-Chloroethyl)trimethyl-ammonium chloride	999-81-5	N		N	
Cyclohexanone	108-94-1	N		N	
N,N-dicyclohexyl thiourea	1212-29-9	N		N	
Trisodium EDTA trihydrate	150-38-9	N		N	
Ephidrine sulphate	134-72-5	N		N	
Erythromycin stearate	643-22-1	N		N	
Fluometron	2164-17-2	N		N	
Phenanthrene	85-01-8	**Y(S9)**[a]	$3.16x10^{-6}$	N	
III. Non-genotoxic carcinogens					
D-limonene	5989-27-5	N		N	
Di-(2-ethylhexyl)phthalate	117-81-7	N		N	
Amitrole	61-82-5	N		N	
Tert-butyl alcohol	75-65-0	N		N	
Diethanolamine	111-42-2	N		N	
Melamine	108-78-1	N		N	
Methyl carbamate	598-55-0	N		N	
Progesterone	57-83-0	N		**Y**	$3.16x10^{-6}$
Pyridine	110-86-1	N		N	
Tris(2-ethylhexyl)phosphate	78-42-2	N		N	
Hexachloroethane	67-72-1	N		**Y**	$3.16x10^{-4}$

[a] Compounds that are activated by S9 mixture but already show some activity without the addition of S9 mixture.

Table 4. Results from the Vitotox™ and RadarScreen validation with respect to the ECVAM compound list.

	Vitotox™ (%)	RadarScreen (%)	Vitotox™ + RadarScreen (%)	N
Sensitivity	70 (14/20)	70 (14/20)	85 (17/20)	20
Specificity	93 (39/42)	83 (35/42)	81 (34/42)	42
Predictivity	85 (53/62)	79 (49/62)	82 (51/62)	62

Table 5. Results from the additional 192 compounds in the Vitotox™ and RadarScreen assay and available data for the Ames assay and in vitro mammalian genotoxicity tests. In this table the genotoxic compounds are shown.

Chemical	CAS number	Further information	Vitotox™	LEC (mol/l)	Radar Screen	LEC (mol/l)	Ames	In vitro mam. genotoxicity	SCE	MN	CA
I. Ames positive compounds											
1-Nitropyrene	5522-43-0		Y	$3.16x10^{-6}$	Y(S9)	$3.16x10^{-4}$	Y [24]	Y [24]	Y IARC		Y IARC
2,4-Dinitrophenol	51-28-5		Y	$3.16x10^{-5}$	Y	$3.16x10^{-5}$	Y	Y [25]			Y [25]
2,7-Dinitrofluorene	5405-53-8		Y	$3.16x10^{-6}$	Y	$1.00x10^{-3}$	Y [26,27]	Y [27]	Y [27]		
2-Amino-3-methyl-3H-imidazo-[4,5-f]-quinoline	76180-96-6		Y(S9)[a]		N		Y [28]	Y [24], IARC		Y [24], IARC	
2-Hydroxy-estradiol	362-05-0	Catechol estrogen	Y(S9)	$1.00x10^{-4}$	Y	$3.16x10^{-5}$	Y [12]	Y/N [12, 29]	Y/N [12, 29]	Y [29]	Y [12]
2-Hydroxy-estrone	362-06-1	Catechol estrogen	Y(S9)	$3.16x10^{-5}$	Y	$3.16x10^{-5}$	Y [12]	Y/N [12, 29]	N [12,29]	N [29]	Y [12]
2-Methoxy-estradiol	362-07-2	Catechol estrogen	Y(S9)	$3.16x10^{-5}$	Y	$3.16x10^{-5}$	Y [12]	Y [12]			Y [12]
2-Methoxy-estrone	362-08-3	Catechol estrogen	N		Y	$3.16x10^{-5}$	Y [12]	Y/N [12]			N [12]
3-Methylcholanthrene	56-49-5		Y	$3.16x10^{-5}$	Y	$1.00x10^{-3}$	Y [24]	Y/N [24, 30]	Y [24]	Y [24]	Y [24]
4-Hydroxy-estradiol	5976-61-4	Catechol estrogen	N		Y	$3.16x10^{-5}$	Y [12]	Y/N [12, 29]	Y/N [12, 29]		Y [12]
4-Hydroxy-estrone	3131-23-5	Catechol estrogen	Y(S9)	$3.16x10^{-6}$	Y	$1.00x10^{-5}$	Y [12]	Y/N [12, 29]	Y/N [12, 29]		Y [12]
4NQO	56-57-5		Y	$3.16x10^{-6}$	Y	$\leq 1.00x10^{-6}$	Y[8, 18, 24]	Y [18, 24]	Y [18, 24]	Y [24]	Y [24]
Dacarbazine	224-396-1		Y	$3.16x10^{-6}$	Y	$1.00x10^{-3}$	Y [24]	Y/N [30, 31], IARC	Y IARC	Y/N [24, 30]	
Dantrolene	14663-23-1		Y	$3.16x10^{-5}$	N		Y [32]	ND			
Doxorubicin	25316-40-9	TOPO	Y		Y	$1.00x10^{-3}$	Y [18, 26]	Y [18]	Y [18]	Y [18]	Y [18]
Ellipticin	519-23-3	TOPO	Y	$3.16x10^{-6}$	Y(S9)[a]	$3.16x10^{-5}$	Y	ND			
Hydralazine	304-20-1	Intercalating	N		N		Y [33], IARC	N[34]		N [34]	
Hydrogen peroxide	7722-84-1	ROS	Y		Y	$3.16x10^{-4}$	Y [8, 18]	Y[8, 18, 24]	Y [18]	Y [18]	Y [8, 18, 24]
β-Naphthoflavone	6051-87-2		Y	$3.16x10^{-5}$	N		Y	N[35]			
Melphalan	148-82-3		N		N		Y [24, 26]	Y[24]			Y [24]
Methampyrone	68-89-3	TOPO, intercalating	Y	$1.00x10^{-4}$	N		Y [36]	ND			
Nitrofurantoin	67-20-9	ROS	Y(S9)	$3.16x10^{-6}$	Y	$3.16x10^{-4}$	Y [8, 24]	Y/N[8, 24, 32]	Y [8, 24]	N [8]	Y/N [8, 24]
Org 20494			Y	$7.90x10^{-4}$	Y	$1.00x10^{-3}$	Y	ND			
Org 2249			Y(S9)	$3.16x10^{-5}$	N		Y	ND			
Org 2408	85750-29-4		Y(S9)	$1.00x10^{-5}$	Y(S9)	$1.00x10^{-4}$	Y	ND			
Org 2508			Y	$7.90x10^{-4}$	N		Y	ND			
Org 32018			Y(S9)	$1.00x10^{-5}$	N		Y	ND			
Org 3240			Y(S9)	$3.16x10^{-5}$	Y(S9)	$3.16x10^{-4}$	Y	Y(V79 gene mutation assay)			
Org 4122	67363-15-9		Y	$1.00x10^{-4}$	N		Y	ND			
Org 42671			Y	$3.16x10^{-5}$	Y(S9)[a]	$3.16x10^{-5}$	Y	ND			
Org 4330			Y(S9)	$1.00x10^{-5}$	N		Y	ND			
Org 5694			Y(S9)[a]	$3.16x10^{-6}$	Y(S9)[a]	$3.16x10^{-5}$	Y	ND			
Org 5695			Y(S9)[a]	$3.16x10^{-6}$	Y	$3.16x10^{-4}$	Y	ND			
Org 5697			N		N		Y	ND			
Org 5710			Y	$1.00x10^{-4}$	N		Y	ND			
Org 5741			Y	$7.90x10^{-4}$	N		Y	ND			

Table 5. Continued

Chemical	CAS number	Further information	Vitotox™	LEC (mol/l)	Radar Screen	LEC (mol/l)	Ames	In vitro mam. genotoxicity	SCE	MN	CA
Org 5784			Y(S9)[a]	$7.90x10^{-4}$	N		Y	ND			
Org 5796			Y(S9)[a]	$7.90x10^{-4}$	N		Y	ND			
Org 5867			Y(S9)	$1.00x10^{-4}$	N		Y	ND			
Org 5907			Y	$7.90x10^{-4}$	N		Y	ND			
Org 7797			Y(S9)	$3.16x10^{-5}$	Y(S9)	$3.16x10^{-5}$	Y	N	N		
Org 9063			Y(S9)	$1.00x10^{-4}$	N		Y	N	N		
Org 9150			Y	$3.16x10^{-5}$	Y	$1.00x10^{-3}$	Y	ND			
Org 9250			Y	$1.00x10^{-5}$	Y (S9)[a]	$3.16x10^{-5}$	Y	ND			
Org 9252			Y(S9)	$1.00x10^{-4}$	N		Y	ND			
Salicylamide	65-45-2		Y(S9)	$1.00x10^{-4}$	N		Y	Y [30]			
Tacrine	1684-40-8		N		Y	$1.00x10^{-3}$	Y [37]	Y/N [38, 39], Y in UDS test		N [38]	N [38]
UK-57400	99470-74-3		Y	$2.50x10^{-4}$	N		Y	ND			
Uramustine	66-75-1		N		Y	$3.16x10^{-4}$	Y [26]	ND			
II. Ames equivocal and in vitro mammalian genotoxic											
5-Fluorouracil	51-21-8	Anti-metabolite	N		Y	$3.16x10^{-6}$	Y/N [18, 24]	Y [18, 24]	Y [18, 24]	Y [18, 24]	Y [18, 24]
III. Ames negative or ND and in vitro mammalian genotoxic											
16α-Hydroxy-estrone	18186-49-7	Estrogen	N		Y	$3.16x10^{-5}$	N [12]	Y/N [12, 29]	Y [29]	N [29]	N [12]
17α-Methyltestosterone	58-18-4	Androgen	N		Y	$3.16x10^{-4}$	ND ICSAS	Y/N [12]	Y/N [12]		
Acetaminophen	103-90-2		N		N		N [24, 32]	Y/N [14, 24, 32]		Y [14, 24]	Y [14]
Allylestrenol	432-60-0	Progestin	N		Y	$3.16x10^{-5}$	ND	Y/N [12]		Y/N [12]	
Bromobenzene	108-86-1	ROS	N		N		N [26]	Y/N [40]	N [40]		Y [40]
Canrenoate K+	2181-04-6	Progestin	N		N		N [12]	Y/N [12]	Y/N [12]	Y/N [12]	
Carbon tetrachloride	56-23-5	ROS	N		N		N [24, 26]	Y/N [24]		Y [24]	N [24]
Chlormadinone	302-22-7	Progestin	N		Y	$1.00x10^{-5}$	N IARC	Y/N [12]		Y [12]	N [12]
Chlormadinone acetate	302-22-7	Progestin	N		Y	$1.00x10^{-5}$	ND	Y/N [12]	Y/N [12]		
Chlorpromazine	69-09-0	N-Dialkyl	Y(S9)	$3.16x10^{-6}$	Y	$1.00x10^{-3}$	N [41]	Y/N [41, 42]	Y [41, 42]		
Chlorprothixene citrate	113-59-7	N-Dialkyl	N		Y	$1.00x10^{-3}$	N [43]	Y [42]	Y[42]		
Clomiphene	50-41-9	SERM, N-Dialkyl	N		Y	$3.16x10^{-4}$	ND	Y [32]			
Colchicine	64-86-8	Spindle poison	N		N		N [8, 18]	Y/N [8, 18]	N [8]	Y [18]	Y [8]
Cyproterone acetate	427-51-0	Progestin	N		Y(S9)	$3.16x10^{-5}$	N [12]	Y/N [12]	Y [12]	Y/N [12]	N [12]
Cytarabine	147-94-4	Anti-metabolite	N		N		N [44]	Y [28, 31]		Y [31]	
Dexamethasone	50-02-2	Glucocorticoid	N		N		N [12]	Y [12]	Y [12]	Y [12]	Y [12]
Diclofenac	15307-79-6		N		Y	$1.00x10^{-4}$	N [32]	Y/N [32, 45, 46]	N [46]		
Diethylstilbestrol		Estrogen	N		Y	$3.16x10^{-5}$	N CPDB	Y [28]		Y [28]	
Diethylthio carbamic acid	148-18-5		N		N		N [24]	Y/N [24]			
Dihydroergotamine mesylate	511-12-6		N		Y	$1.00x10^{-3}$	N [32]	Y [32]			Y [32]
Drospirenone	67392-87-4	Progestin	N		Y	$1.00x10^{-5}$	N [12]	Y/N [12, 47]	Y/N [12]	N [12]	N [12]
Dydrogesterone	152-62-5	Progestin	N		Y	$1.00x10^{-5}$	ND	Y/N [12, 48]		Y/N [12]	
Equilin	474-86-2	Estrogen	Y(S9)	$3.16x10^{-5}$	Y	$1.00x10^{-5}$	ND	Y/N [12, 49, 50]	N [12]		

Table 5. Continued

Chemical	CAS number	Further information	Vitotox™	LEC (mol/l)	Radar Screen	LEC (mol/l)	Ames	In vitro mam. genotoxicity	SCE	MN	CA
Equilin-7a-methyl		Estrogen	Y(S9)	$1.00x10^{-4}$	Y	$1.00x10^{-5}$	ND	Y [50]			
Estradiol-17α	57-91-0	Estrogen	N		Y	$3.16x10^{-6}$	ND	Y/N [12, 51]			N [12]
Estradiol-17β	50-28-2	Estrogen	N		Y	$1.00x10^{-5}$	N [12, 24, 52]	Y/N [12, 24, 52]	Y/N[12,24,52]	Y/N[12,24,52]	Y/N[12,24]
Estriol	50-27-1	Estrogen	N		Y	$1.00x10^{-5}$	ND	Y/N [12]	Y [12]	N [12]	Y/N [12]
Estrone (E1)	53-16-7	Estrogen	N		Y	$3.16x10^{-5}$	ND	Y/N [12]	Y/N [12]	Y [12]	Y [12]
Ethinylestradiol-17β	57-63-6	Estrogen	N		Y	$1.00x10^{-6}$	N [12,24,53,54]	Y/N	Y/N	Y/N	Y/N
Hydrochlorothiazide	58-93-5		N		N		N [24], CPDB	Y/N [24, 55]	Y [24, 55]	N [24, 55]	
Hydroxychloroquine sulfate	58-93-5		N		Y	$1.00x10^{-3}$	ND	Y [56]	Y [56]		
ICI 164.384	98007-99-9	Anti-estrogen	N		Y(S9)	$1.00x10^{-5}$	ND	Y [57]			Y [57]
Imipramine HCl	113-52-0	N-Dialkyl	N		N		N [58]	Y/N [30, 32, 37]			
Levonorgestrel	797-63-7	Progestin	N		Y	$1.00x10^{-3}$	N [12]	Y/N [12]	Y [12]	N [12]	N [12]
Medroxyprogesterone acetate	520-85-4	Progestin	N		Y	$1.00x10^{-5}$	N	Y/N [12]	Y/N [12]	N [12]	N [12]
Megestrol acetate	595-33-5	Estrogen	N		Y	$1.00x10^{-5}$	ND	Y/N[12]		Y [12]	N [12]
Ment-bucyclate		Androgen	N		N		N	Y			Y
Mestranol	72-33-3	Estrogen	N		NA		N [12]	Y/N [12, 59]	Y/N [12, 59]	Y [12]	Y/N [12, 59]
Methotrexate	59-05-2	Anti-metabolite	N		Y	$1.00x10^{-5}$	N [24, 26]	Y [24, 60]	Y [24]	Y [60]	Y [24]
Moxestrol	34816-55-2	Estrogen	N		Y	$1.00x10^{-5}$	ND	Y [12]			Y [12]
Norethisterone	68-22-4	Progestin	N		Y	$3.16x10^{-5}$	N [12]	Y/N [12]	Y/N [12]	N [12]	Y/N [12]
Norethynodrel	68-23-5	Progestin	N		Y	$1.00x10^{-5}$	N [12, 61]	Y/N [12, 62]	Y/N [12, 62]		Y [62]
Noscapine HCl	912-60-7	Aneugen	N		N		N [30]	Y [8]			Y[8]
Org 10325			N		N		N	Y	Y		
Org 30029			Y	$1.00x10^{-4}$	Y	$1.00x10^{-3}$	N	Y	Y		
Org 30251			Y(S9)	$1.00x10^{-4}$	Y	$1.00x10^{-3}$	N	Y	Y		
Org 39735	319003-75-3	Androgen	N		Y	$3.16x10^{-5}$	N	Y			Y
Org 4433	68598-93-6	Progestin	N		Y	$1.00x10^{-5}$	N	Y			
Oxymethalone	434-07-1	Androgen	N		Y	$1.00x10^{-6}$	N[12]	Y/N [12]	Y/N [12]	N [12]	N [12]
Rifampicin	13292-46-1		N		Y	$3.16x10^{-5}$	N [63]	Y [30]			Y [30]
Rotenone	83-79-4		N		N		N [24]	Y/N [24]	Y [24]	Y [24]	N [24]
Stanozolol	10418-03-8	Androgen	N		Y	$1.00x10^{-6}$	ND	Y/N [12]	Y/N [12]		
Sulfinpyrazone	57-96-5		N ICSAS		Y	$3.16x10^{-5}$	N	Y			
Tamoxifen	10540-29-1	Non-steroidal anti estrogen, N-Dialkyl	N		N		N [32]	Y [24, 37, 57]		Y [24, 57]	
Testosterone	58-22-0	Androgen	N		Y	$1.00x10^{-5}$	ND	Y/N [12, 64]	Y/N [12, 64]	N [12]	
Tolcapone	134308-13-7	Catechol	N		Y	$1.00x10^{-5}$	N [25]	Y/N [32]	Y/N [32]		
Trenbolone	10161-33-8	Androgen	N		Y	$1.00x10^{-4}$	N [12], IPCS	Y/N [12], IPCS	N [12]	Y/N [12], IPCS	N [12]

Abbreviations: CPDB=carcinogenic potency project database; IARC=international agency for research on cancer database; ICSAS=international agency for computional safety database (FDA database); IPCS=international programme on chemical safety database; ROS=reactive oxygen species; SERM=selective estrogen receptor modulator, TOPO=topoisomerase inhibitor;[a] Compounds that are activated by S9 mixture but already show some activity without the addition of S9 mixture.

Table 6. Results from the additional 192 compounds in the Vitotox™ and RadarScreen assay and available data for the Ames assay and in vitro mammalian genotoxicity tests. The non-genotoxic compounds, compounds from which no genotoxicity data are available, and compounds that are equivocal for genotoxicity are summarized in this table.

Chemical	CAS number	Further information	Vitotox™	LEC (mol/l)	Radar Screen	LEC (mol/l)	Ames	In vitro mam. genotoxicity	SCE	MN	CA
2,5-Hexanedione	110-13-4		N		N		ND ICSAS	ND			
7α-Methylnorthisterone		Androgen	N		Y	$3.16x10^{-4}$	ND	ND			
Acetylsalicylic acid	50-78-2		N		N		N [24, 32]	N [24, 28, 32]		N [28]	
Aminophylline	317-34-0		N		N		ND	N [30]			
Amiodarone	19774-82-4	N-dialkyl	N		N		N [12]	N [32]			
Antazoline mesylate	2508-72-7		N		N		ND	ND			
Atamestane	96301-34-7	Anti-estrogen	N		Y	$1.00x10^{-5}$	N [12]	N [12, 53]		N [12]	N [53]
Atropine sulfate	5908-99-6		N		N		N [55]	ND			
Bishydroxycoumarin	66-76-2		N		Y	$1.00x10^{-4}$	N[24]	N [24]			N [24]
CERM 11884			N		N		N	N	N		
CERM 13061			N		Y	$3.16x10^{-4}$	N	ND			
Clozapine	5786-21-0		N		N		N [32]	N [55]	N [55]		
Corticosterone	50-22-6	Glucocorticoid	N		Y	$3.16x10^{-5}$	ND	ND			
Cortisol	50-23-7	Glucocorticoid	N		N		ND[65]	N [12]	N [12]		
Dehydroepiandrosterone	53-43-0	Androgen precursor	Y	$1.00x10^{-4}$	Y	$3.16x10^{-6}$	N [28]	N [12]	N [12]		
Dimethisterone	79-64-1	Progestin	N		Y	$3.16x10^{-6}$	ND	N [12]			N[12]
Dopamine	62-31-7	Catechol amine	N		N		N ICSAS	N ICSAS			
Erythromycin	114-07-8		N		N		N [24, 32]	N [24, 32]	N [24, 32]		N [32]
Ethacrynic acid	58-54-8		N		N		ND	ND			
Ethionine	67-21-0		Y	$1.00x10^{-4}$	N		N [24, 26]	N [30, 34]			
Ferrous sulphate	7782-63-0		N		N		ND	N [30]			
Fluoxymesterone without 17α-methyl	76-43-7	Androgen	N		Y	$3.16x10^{-4}$	ND	ND			
Flutamide	13311-84-7	Non-steroidal anti-androgen	N		N		N ICSAS	N [66]		N [66]	
Furafylline	80288-49-9		N		N		ND	ND			
Gentamicin A	1405-41-0		N		N		N ICSAS	N ICSAS			
Hexachlorobutadiene	87-68-3		N		N		N [24]	N [24]			N [24]
Indomethacin	53-86-1		N		N		N [32]	N [30]			
Iodoacetate	305-53-3		N		N		N [18]	N [18, 32]	N [18]		Y [18]
Iproniazid	54-92-2		N		Y	$3.16x10^{-5}$	N [33]	ND			
Isoprenaline	51-30-9		N		Y(S9)	$3.16x10^{-4}$	ND	ND			
Ketoconazole	65277-42-1		N		N		N [32]	ND			
Labetalol	32780-64-6		N		N		N [32]	ND			

Table 6. Continued

Chemical	CAS number	Further information	Vitotox™	LEC (mol/l)	Radar Screen	LEC (mol/l)	Ames	In vitro mam. genotoxicity	SCE	MN	CA
L-DOPA	79559-97-0		N		N		N [32]	N [40]			
Lilopristone with additional carbon in 17α chain		Anti-progestin	Y(S9)	$1.00x10^{-5}$	Y(S9)[a]	$3.16x10^{-6}$	N[62]	ND			
Methadone	76-99-3	N-Dialkyl	N		N		ND ICSAS	N [67]			N [67]
Naphazoline nitrate	5144-52-5		N		N		ND	ND			
N-Ethylmaleimide	128-53-0	Thiol reactive	N		N		ND	ND			
Onapristone	96346-61-1	Anti-progestin	N		Y(S9)[a]	$3.16x10^{-5}$	N [12]	N [12]		N [12]	N [12]
Org 10490			N		N		N	ND			
Org 13011			N		N		N	ND			
Org 20091			N		N		N	N (V79 gene mutation test)			
Org 20223			N		N		N	N (V79 gene mutation test)			
Org 20241	145261-31-0		N		N		N	ND			
Org 20350			N		N		N	N (V79 gene mutation test)			
Org 20660			N		Y(S9)	$1.00x10^{-3}$	N	ND			
Org 30189			N		N		N	ND			
Org 30535			N		N		N	N	N		
Org 30659	110072-15-6	Progestin	N		Y(S9)[a]	$1.00x10^{-5}$	N	N	N		
Org 30701			N		N		N	N	N		
Org 31710	118968-41-5	Anti-progestin	N		Y	$3.16x10^{-4}$	N	ND			
Org 32608			N		N		N	ND			
Org 32782			N		Y	$1.00x10^{-3}$	N	N			N
Org 3362			N		N		N	ND			
Org 34037	218899-99-1		N		N		N	N			N
Org 34694	160242-34-2	Estrogen	N		Y	$3.16x10^{-4}$	N	N			N
Org 34850	162607-84-3	Anti-glucocorticoid	N		N		N	N			N
Org 34901	177900-66-2	Progestin	N		Y	$3.16x10^{-5}$	N	N			N
Org 37445		Estrogen	N		Y	$1.00x10^{-5}$	N	ND			
Org 4060		Progestin	N		N		N	N			N
Org 42788		Estrogen	N		Y	$3.16x10^{-6}$	N	ND			
Org 4428	135928-30-2		N		N		N	N			
Org 4874			N		N		N	ND			
Org 5168			N		N		N	ND			
Org 7258			N		Y	$1.00x10^{-5}$	N	ND			
Org 9340			N		Y(S9)[a]	$3.16x10^{-4}$	N	ND			
Orphenadrine citrate	4682-36-4		N		N		ND	ND			
Papaverine HCl	61-25-6		N		N		ND ICSAS	ND ICSAS			

Table 6. Continued

Chemical	CAS number	Further information	Vitotox™	LEC (mol/l)	Radar Screen	LEC (mol/l)	Ames	In vitro mam. genotoxicity	SCE	MN	CA
Perhexiline	6724-53-4		N		N		N [68]	ND			
Perphenazine	58-39-9		N		N		ND ICSAS	N [42]			
Phentolamine mesylate	50-60-2		N		N		N ICSAS	N [30]			
Propylmesterolone	79243-67-7	Progestin	N		Y	$1.00x10^{-5}$	N [12]	N [12]		N [12]	N [12]
Quinidine	56-54-2		N		N		ND	ND			
Quinidine sulfate	6591-63-5		N		N		ND	ND			
R1881	965-93-5	Androgen	N		Y	$3.16x10^{-4}$	ND	ND			
Raloxifen	84449-90-1	SERM	N		Y	$3.16x10^{-4}$	N [32]	N [32]			
Reserpine	50-55-5		N		N		N [24]	N [24, 30]	N [24]		N [24]
RU 58668	151555–47–4	Anti-estrogen	N		Y	$3.16x10^{-4}$	ND	ND			
Strychnine	57-24-9		N		N		ND	ND			
Sulfamoxole	729-99-7		N		N		ND	ND			
Sulfaphenazole	526-08-9		N		Y	$1.00x10^{-3}$	ND	ND			
Tularik 0191317		LXR/PXR agonist	N		N		ND	ND			
Equivocal for genotoxicity											
Digoxin	20830-75-5		N		N		Y/N[65]	ND			
Org 30002			N		N		Y/N	N			
Org 9217			N		N		Y/N	ND			
Org 9935			N		N		Y/N	N			

[a]Compounds that are activated by S9 mixture but already show some activity without the addition of S9 mixture; Abbreviations: ICSAS=international agency for computional safety database (FDA database); SERM=selective estrogen receptor modulator, TOPO = topoisomerase inhibitor

Table 7. Overview of the in-house compound codes and the corresponding chemical names.

Org code	Chemical name
CERM 11884	N-(2,6-Dimethylphenyl)-beta-[(2-methylpropoxy)methyl]-N-(phenylmethyl)-1-pyrrolidineethanamine hydrochloride
CERM 13061	*trans*-D,L-N-(1,3-Benzodioxol-5-yl)-2-[(cyclohexyloxy)methyl]-1-methyl-N-(phenylmethyl)-3-piperidinamine hydrochloride(1:1) salt
Org 10325	N-Hydroxy-5,6-dimethoxy-1H-indene-2-carboximidamide hydrochloride
Org 10490	6,7,8,9-Tetrahydro-7-methyl-5H-dibenz[b,i][1,6]oxazecine(Z)-2-butenedioate (1:1)
Org 13011	1-[4-[4-[4-(Trifluoromethyl)-2-pyridinyl]-1-piperazinyl]butyl]-2-pyrrolidinone(E)-2-butenedioate (1:1)
Org 20091	5-Chloro-4-(1,2,5,6-tetrahydro-1-methyl-3-pyridinyl)-2-thiazolamine(E)-2-butenedioate (2:1)salt
Org 20223	2-Amino-N-(2-phenylethyl)-N-propyl-5-thiazoleethanamine (E)-2-butenedioate (1:1)salt
Org 20241	N-Hydroxy-4-(3,4-dimethoxyphenyl)-2-thiazolecarboximidamide
Org 20350	4-(1-Butyl-1,2,5,6-tetrahydro-3-pyridinyl)-5-chloro-2-thiazolamine(Z)-2-butenedioate (1:1) salt
Org 20494	4,5-Dihydro-5-methyl-6-[6-[2-(1-piperidinyl)ethoxy]benzo[b]thien-2-yl]-3(2H)-pyridazinonehydrochloride
Org 20660	2-Chloro-11-(1,2,5,6-tetrahydro-1-methyl-3-pyridinyl)dibenzo[b,f][1,4]thiazepine (E)-2-butenedioate (1:1) salt
Org 2249	2,3,9,13b-Tetrahydro-2-methyl-1H-dibenz[c,f]imidazo[1,5-a]azepine
Org 2408	1,2,3,4,10,14b-hexahydro-2,7-dimethyldibenzo(c,f)pyrazino(1,2-a)azepine^(z)-2-butenedioate (1:1)
Org 2508	1-(n-hydroxyamidino)-benzocyclobutenehydrochloride
Org 30002	5,6,7,8-Tetrahydro-6-methyldibenz[b,h][1,5]oxazonine hydrochloride hemihydrate
Org 30029	N-Hydroxy-5,6-dimethoxybenzo[b]thiophene-2-carboximidamide hydrochloride
Org 30189	1-[2-Chloro-3-(2-methylpropoxy)propyl]pyrrolidinemonohydrochloride
Org 30251	2,3,4,5,6,7-Hexahydro-N-hydroxy-1H-benz[e]indene-2-carboximidamide hydrochloride
Org 30535	cis-1,2,3,12b-Tetrahydro-2,7-dimethyl-3aH-dibenz[2,3:6,7]oxepino[4,5-c]pyrrol-3a-ol Z)-2-butenedioate (1:1)
Org 30701	1-[1-[(2-Methylpropoxy)methyl]-2-[[1-(1-propynyl)cyclohexyl]oxy]ethyl]pyrrolidinehydrochloride
Org 31710	(6beta,11beta,17beta)-11-[4-(Dimethylamino)phenyl]-4',5'-dihydro-6-methylspiro[estra-4,9-diene-17,2'(3'H)-furan]-3-one
Org 32018	1-[2-(Phenylmethoxy)ethyl]-4-(3-phenylpropyl)piperazine dihydrochloride
Org 3240	n-hydroxy-3,4-dimethoxybicyclo(4.2.0)octa-1,3,5-triene-7-carboximidamide^hydrochloride
Org 32608	2-[2-(4-Chlorophenoxy)phenyl]-4,5-dihydro-1H-imidazole[Z]-2-butenedioate (1:1)
Org 32782	2-(3,4-Dichlorophenoxy)benzenemethanaminehydrochloride
Org 3362	n-hydroxy-3,5-dimethylbicyclo(4.2.0)octa-1,3,5-triene-7-carboximidamide^hydrochloride
Org 34037	R(-)-6-(4-Chlorophenyl)-2,3,5,6-tetrahydroimidazo[2,1-a]isoquinoline(E)-2-butenenedioate (1:1) salt
Org 34694	(7alpha,11E,17alpha)-11-Ethylidene-17-hydroxy-7-methyl-19-norpregn-4-en-20-yn-3-one
Org 34850	(11beta,17alpha)-11-[4-(Dimethylamino)phenyl]-17-hydroxy-21-[4-(methylsulfonyl)phenyl]-19-norpregna-4,9-dien-20-yn-3-one
Org 34901	(11beta,17alpha)-17-Hydroxy-3-oxo-11-(1-propynyl)-19-norchola-4,20-dien-24-oicacid deltalactone
Org 37445	(3alpha,11beta,17alpha)-11-(2-Propenyl)-19-norpregn-5(10)-en-20-yne-3,17-diol
Org 39735	(7alpha,17beta)-7-Ethyl-17-hydroxyestra-4,14-dien-3-one
Org 4060	(11beta,17alpha)-11-Ethyl-17-hydroxy-19-norpregn-4-en-20-yn-3-one
Org 4122	N-Hydroxy-3,4-dimethoxybenzenepropanamide
Org 42671	2,8-dihydroxy-10 chloro-11H-[b]-benzofluorene
Org 42788	(3alpha,7alpha,11beta,16alpha,17alpha)-7,11,16-Trimethyl-19-nor^pregn-5(10)-en-20-yne-3,17-diol
Org 4330	n-hydroxy-3,4-dimethoxybicyclo(4.2.0)octa-1,3,5-triene-7-carboxamide
Org 4428	cis-1,3,4,13b-Tetrahydro-2,10-dimethyldibenz[2,3:6,7]oxepino[4,5-c]pyridin-4a(2H)-ol
Org 4433	(11beta,17alpha)-11-Ethynyl-17-hydroxy-19-norpregn-4-en-20-yn-3-one
Org 4874	n,3,4-trimethoxybenzenepropanamide
Org 5168	3a,4,9,9a-Tetrahydro-6,7-dimethoxy-1H-benz[f]isoindole-1,3(2H)-dione
Org 5694	N-(Acetyloxy)-3,4-dimethoxybicyclo[4.2.0]octa-1,3,5-triene-7-carboximidamide

Table 7. Continued

Org code	Chemical name
Org 5695	3,4-Dimethoxy-N-[[(methylamino)carbonyl]oxy]bicyclo[4.2.0]octa-1,3,5-triene-7-carboximidamide
Org 5697	3-(3,4-Dimethoxybicyclo[4.2.0]octa-1,3,5-trien-7-yl)-5-(trifluoromethyl)-1,2,4-oxadiazole
Org 5710	N,N'-Dihydroxy-3,4-dimethoxybicyclo[4.2.0]octa-1,3,5-triene-7-carboximidamide
Org 5741	N-Hydroxy-3,4-dimethoxy-N-methylbicyclo[4.2.0]octa-1,3,5-triene-7-carboximidamide hydrochloride
Org 5784	N-Hydroxy-3,4-dimethoxy-N'-methylbicyclo[4.2.0]octa-1,3,5-triene-7-carboximidamidehydrochloride
Org 5796	N-Hydroxy-3,4-dimethoxy-7-methylbicyclo[4.2.0]octa-1,3,5-triene-7-carboximidamide (Z)-2-butenedioate
Org 5867	N,3,4-Trimethoxybicyclo[4.2.0]octa-1,3,5-triene-7-carboximidamide hydrochloride
Org 5907	cis-N-Hydroxy-3,4-dimethoxy-8-methylbicyclo[4.2.0]octa-1,3,5-triene-7-carboximidamidehydrochloride
Org 7258	2-(4-Chlorophenyl)-4-phenyl-1H-indene-1,3(2H)-dione
Org 7797	(16alpha,17beta)-17-(Methylamino)estra-1,3,5(10)-triene-3,16-diol(Z)-2-butenedioate
Org 9063	3,4-Dihydro-N-hydroxy-6,7-dimethoxy-2-naphthalenecarboximidamide hydrochloride(1:1)salt
Org 9150	3-(5,6-Dimethoxy-1H-inden-2-yl)-2-propenenitrile
Org 9217	(E)-N-Hydroxy-3-(5,6-dimethoxy-2-benzofuranyl)-2-propenimidamidehydrochloride
Org 9250	Sodium 5,6-dimethoxy-N-hydroxybenzo[b]thiophene-2-carboxamidemonohydrate
Org 9252	4-Chloro-N-hydroxy-5,6-dimethoxybenzo[b]thiophene-2-carboximidamidehydrochloride
Org 9340	DL-(1alpha,2beta,4alpha)-4-([1,1'-Biphenyl]-4-ylmethyl)-2-[(1,1-dimethylethyl)amino] cyclohexanolmethanesulfonate (1:1) salt
Org 9935	4,5-Dihydro-6-(5,6-dimethoxybenzo[b]thien-2-yl)-5-methyl-3(2H)-pyridazinone

Validation of the Vitotox™ assay against Ames results

The results from the Vitotox™ assay were compared to the Ames data obtained from in-house experiments or from the literature (Table 8). This resulted in a sensitivity of 86% (42/49). The number of false positive results was low, as only six compounds were identified, which leads to a specificity of 94% (90/96). The correlation of the Vitotox™ assay with respect to Ames results was 91% (132/145).

The false positive and false negative results are summarized in Table 9. Of the six false positive compounds five compounds were also positive in the RadarScreen assay. Furthermore, three of these compounds being chlorpromazine HCl, Org 30029 and Org 30251 gave a positive result for in vitro mammalian genotoxicity. This means that the number of irrelevant positive results is even lower than the six compounds and is in the range of 1-3 out of 145 compounds.

For two of the five false negative compounds, cyclophosphamide and melphalan, it is known that a SOS response is not activated in *Salmonella* [4, 13]. For the other compounds no clear explanation can be given for the negative result. Overall, the results suggest that the Vitotox™ assay is an early screening tool that predicts Ames results very well. Moreover, several compounds that were positive in the RadarScreen and/or in in vitro mammalian genotoxicity assays were detected in the Vitotox™ assay.

Table 8. Validation of the Vitotox™ and RadarScreen assays with respect to the additional set of 192 compounds. Vitotox™ and RadarScreen results were, respectively, compared with Ames or in vitro mammalian genotoxicity data.

	Vitotox™-Ames		RadarScreen-in vitro mammalian genotoxicity	
	Score (%)	**N**	**Score (%)**	**N**
Sensitivity	86 (42/49)	49	77 (59/77)	77
Specificity	94 (90/96)	96	74 (34/46)	46
Correlation	91 (132/145)	145	76 (93/123)	123

Validation of the RadarScreen assay against in vitro mammalian genotoxicity tests (in vitro SCE, chromosome aberration and micronucleus tests)

The RadarScreen assay was evaluated as a pre-screen for chromosome damage by comparing the results with the in vitro mammalian genotoxicity data that were available for the additional set of 192 compounds. The sensitivity, specificity and correlations are presented in Table 8. The sensitivity was 77% (59/77), the specificity 74% (34/46), and the correlation 76% (93/123). The compounds that had a false positive or false negative result are summarized in Table 10.

One of the false positive results, Org 7797, had clearly genotoxic properties as this compound was positive in the Ames and Vitotox™ assay. Org 32782 had structural alerts for genotoxicity, but no positive results were observed in other genotoxicity assays. Bishydroxycoumarin (dicumarol) induces oxidative stress which might lead to genotoxic effects. However, also in this case other tests did not show any genotoxicity. The remaining compounds with a false positive result were all steroidal compounds, except the anti-estrogen raloxifen. For structurally related compounds genotoxic potential has been reported.

Of the false negative results bromobenzene and carbon tetrachloride are both very lipophylic compounds. These compounds do not dissolve properly in growth medium and are, therefore, in our view of doubtful value. However, these compounds were not skipped from analysis. Acetaminophen and dexamethasone probably show genotoxic effects due to an irrelevant mechanism of cytotoxicity [14]. In our assay system no genotoxic effect was measured. The aneugenic compound colchicine was not detected in the RadarScreen assay. As the aneugenic compounds taxol (ECVAM compound list) and noscapine HCl were also not detected in the RadarScreen assay this might suggest that compounds with an aneugenic mode of action are not or difficult to detect in the RadarScreen assay. Despite the structural resemblance between both anti-estrogens raloxifen and tamoxifen, one appeared false positive and the other false negative. Ment-bucyclate was thought to be a false negative since bucyclate normally should induce clastogenicity. However, it may be that bucyclate is not formed under the conditions in this assay.

Table 9. False positive and false negative results in the Vitotox™ assay with respect to the additional set of 192 compounds after comparison of the results with Ames data. Several of the false positive results have a positive score for genotoxicity in other assays (indicated in bold).

False positive results		**False negative results**	
Chemical	**Futher information**	**Chemical**	**Further information**
Chlorpromazine HCl	RadarScreen positive, clastogenic in vitro	2-Methoxy-estrone	Catechol estrogen
Dehydropiandrosterone	RadarScreen positive, not clastogenic in vitro	4-Hydroxy-estradiol	Catechol estrogen
Ethionine	RadarScreen negative, not clastogenic in vitro	Cyclophosphamide	Gives no SOS response [4]
Org 30029	RadarScreen positive, in vitro SCE positive	Hydralazine	
Org 30251	RadarScreen positive, in vitro SCE positive	Melphalan	SOS response bacteria strain specific
Lilopristone (+17α carbon)	RadarScreen positive	Tacrine	
		Uramustine	

Table 10. False positive and negative results in the RadarScreen assay with respect to the additional set of 192 compounds after comparison of the results with in vitro mammalian genotoxicity data.

False positive results		**False negative results**	
Chemical	**Further information**	**Chemical**	**Further information**
Atamestane		Acetaminophen	Probably positive due to cytotoxicity
Dehydropiandrosterone		Bromobenzene	
Bishydroxycoumarin	ROS formation [69]	Canrenoate K^+	
Dimethisterone		CCl_4	
Org 30659		Colchicine,	Aneugen
Org 31710		Cytarabine	DNA synthesis inhibitor
Org 32782	Structural alerts (ISIS)	DETC	
Org 34694		Dexamethasone	Probably positive due to cytotoxicity
Org 34901		Hydrochlorothiazide	
Org 7797	Ames and Vitotox™ positive	Imipramine HCl	N-Dialkyl, intercalating
Propylmesterolone		Melphalan	Alkylating agent
Raloxifen		Ment-bucyclate	
		Noscapine HCl	Aneugen
		Org 10325	Vitotox™ positive
		Org 39971	
		Quinoline	
		Rotenone	
		Salicylamide	Ames and Vitotox™ positive
		Tamoxifen	

DETC=diethylthiocarbamic acid; ROS=reactive oxygen species

Discussion

The reporter assays

There is a demand for medium- and/or high-throughput genotoxicity tests that can be used for the risk assessment of genotoxicity in the early research phase of drug development. These tests should have a high-throughput and sensitivity, as well as a high specificity. Ideally such a test or combination of tests should predict the Ames test and the occurrence of clastogenicity/aneugenicity. Drug attrition due to these two aspects is relatively high in the pharmaceutical industry. For example, at Organon the attrition rate due to a positive result in the Ames assay during the period of 1970-2003 was 19% (internal data).

In the present study the Vitotox™ and RadarScreen assay were validated as pre-screens for respectively bacterial mutagenicity and in vitro mammalian genotoxicity (chromosome damage). As this is the first paper that describes the RadarScreen assay, experiments were performed to get a first impression about the reproducibility of the RadarScreen assay. Experiments with the reference chemicals MMS and B[a]P showed that the genotoxic effects of these compounds were reproducible. Furthermore, the reproducibility was shown of the method that was used to assess cytotoxicity in the RadarScreen assay. All compounds used in the present study were tested in two independently performed experiments. The results from one representative experiment were shown in this paper. For only seven of the 254 tested compounds inconsistent results were found. These results also indicate that the reproducibility of this assay is good. The seven compounds with inconsistent results were reordered and retested. Based on these results a decision about the genotoxicity in the RadarScreen was made. Although already some experiments were performed to show the reproducibility of the RadarScreen assay, more work needs to be done to measure other robustness parameters, for example, inter-laboratory variation.

It is difficult to select a well-defined compound set for a validation study as especially clastogenicity (mammalian genotoxicity) data in literature are often inconsistent and incomplete, whereas the quality of the data is difficult to judge. Recently a compound list was published as a follow-up of an ECVAM workshop considering ways to reduce the number of irrelevant positive results in in vitro (mammalian) genotoxicity tests. An expert panel identified chemicals that could be used in the evaluation of modified or new in vitro genotoxicity assays. This ‘ECVAM compound list’ was used as a starting point for the validation in the present study.

Validation of the assays with the ECVAM compound list

The results showed that both the Vitotox™ and RadarScreen tests had a sensitivity of 70% (14/20). When both tests were combined the sensitivity increased to 85% (17/20).

This may indicate that the tests can detect compounds with a different mode of action. This is something that was expected as the Vitotox™ is a pre-screen for the Ames test and the RadarScreen assay is able to detect chromosome damage.

Cyclophosphamide was negative in the Vitotox™ assay although it is positive in the Ames test. However, it is known that this compound does not induce a SOS response [4]. Furthermore, dimethylnitrosamine was negative in the Vitotox assay. This might be explained by the fact that this compound needs CYP2E1 for its activation which is only present in low amounts in rat-S9 mixture [11]. On the other hand, this compound was already identified as positive in the RadarScreen assay in the absence of S9 mixture indicating that endogenous metabolism appears active in the yeast cells. Addition of S9 mixture increased the response of dimethylnitrosamine. The presence of endogenous metabolism in yeast is further supported by the fact that benzo[a]pyrene and cyclophosphamide are already positive in the assay in absence of S9 mixture. Nevertheless the activity of these compounds is significantly increased after the addition of S9 mixture. Sodium arsenite, taxol and chloramphenicol, defined in the ECVAM compound list as genotoxic compounds, gave a negative response in the Vitotox™ as well as the RadarScreen assay. As these three compounds are negative (or equivocal) in the Ames assay it is not surprising that these compound show a negative result in the Vitotox™ assay. No clear explanation can be given for the negative results of sodium arsenite and chloramphenicol in the RadarScreen assay. Taxol is an aneugenic compound. The inability to detect aneugenic compounds (like taxol) with the RadarScreen assay is discussed later. The specificity and the predictivity of the two tests combined was 81% (34/42) and 82% (51/62), respectively. These results show that both tests give only a low number of false positive results.

For two of the ECVAM compounds that are in the 'false positive group', tertiary-butylhydroquinone and curcumin, there is still discussion within the ECVAM working group whether these compounds are real genotoxic positives as these compounds give rise to oxidative stress which can result in DNA damage (David Kirkland, personal communication). In the present study tertiary-butylhydroquinone gave a positive response in both the Vitotox™ and RadarScreen assay. Curcumin gave a positive response in only the RadarScreen assay. Moving these two compounds to the list with genotoxic compounds would increase the overall sensitivity (+1%, 86%) and specificity (+7%, 88%) of the combined pre-screen.

Validation of the assays with an additional set of compounds including a large set of steroidal compounds

As the ECVAM compound list is relatively small, an additional set of 192 compounds were tested. These compounds were non-genotoxins and genotoxins with diverse mechanisms of action. However, for a large number of these compounds the mechanism

is still unknown. The compound list also included many in-house compounds from Organon for which genotoxicity data were available. Because a lot of steroids cause genotoxicity in vitro at high (non-physiological) concentrations estrogens, progestagens, androgens, and glucocorticoids were included in this compound list.

The Ames data from the additional list of compounds were in general very consistent and this facilitated a direct comparison of these data with the Vitotox™ and RadarScreen assay results. Difficulties occurred with the in vitro mammalian genotoxicity data, as less information was available. These data were often inconsistent which made a proper judgment difficult. However, as the RadarScreen assay is a test that is meant for pre-screening this test should detect compounds that have the ability to give a positive score in the regulatory genotoxicity tests. This gives the opportunity of de-selecting such compounds or gives time for issue resolution. Therefore, it was decided to define compounds that have a positive score in one mammalian test as genotoxic. However, the goal was not to (re-) classify genotoxic/non-genotoxic compounds.

The results obtained with the additional 192 compounds showed that the Vitotox™ assay had a high correlation (91%; 132/145) with the Ames test results. Similar high correlations between SOS induction and Ames scores were found by others. Quillardet et al. [5] showed that 90% of Ames positive compounds were detected in the SOS chromotest. Von der Hude et al. [15] reported a sensitivity of SOS induction for Ames-positive epoxides of 70%. However, the sensitivity score is dependent on the compound class that is considered. Vasilieva et al. [16] showed that SOS induction correlated closely with Ames results for phenanthrenequinones and fluorenones. However, biphenyls that were positive in the Ames test did not induce the SOS response. Similar results were also reported by Van Gompel et al. [9], who also showed that the sensitivity of the Vitotox™ assay for Ames-positive compounds is low for some compound classes. The specificity, however, was for all compound classes relatively high.

Gentaur (who now distributes the Vitotox™ test) reports that the sensitivity of the Vitotox™ assay for several compounds can be improved by modification of the treatment time from 180 min to 240 min. However, such an increase in sensitivity might result in a decrease of the specificity. More studies are needed to show whether it is preferred to increase the incubation time.

The top dose in the Vitotox™ assay was limited at 0.1 mM. This was not the case for the ECVAM compound list and available Ames data for the additional 192 compounds. Not limiting the top dose might have resulted in a higher sensitivity but also a lower specificity. The reason for using this top dose is that it was expected that the Vitotox™ assay was more sensitive than the Ames assay (with exception of some compound classes that bypass the SOS response).

In the present study also steroidal compounds were tested. Many of these steroids have been reported to show genotoxicity. However, only for catechol estrogens positive results

for bacterial mutagenicity were demonstrated. Of the six catechol estrogens that were tested 2-hydroxy-estradiol, 2-hydroxy-estrone, 2-methoxy-estradiol and 4-hydroxy-estrone showed a positive effect in the Vitotox™ assay. 2-Methoxy-estrone and 4-hydroxy-estradiol did not show such an effect. Although some catechol estrogens gave a negative response in the Vitotox™ assay all catechol estrogens scored positive in the Radarscreen assay. Of the other steroidal compounds tested only four were positive being equilin, 7α-equilin, dehydropiandrosterone and lilopristone (with an additional carbon in the 17α chain). Ames data for equilin and 7α-equilin were not available. However, these two compounds were reported as clastogenic (Table 5). Dehydropiandrosterone is not genotoxic in the regulatory tests, however this compound was positive in the Vitotox™ assay as well as in the RadarScreen assay. No in vitro mammalian genotoxicity data were available for the lilopristone. This compound was positive in the Vitotox™ assay as well as in the RadarScreen assay in the presence of S9 mixture.

The Vitotox™ assay also detected some compounds that were positive in yeast or mammalian cells, but negative in the Ames. The reporter gene recN plays an important role in double strand break repair in bacteria. This might be an explanation for the fact that some clastogenic compounds are also detected in this assay. Of the six false positive results in the Vitotox™ assay, three compounds were positive in the regulatory in vitro mammalian tests. Although some clastogenic compounds were detected in the Vitotox™ assay, the sensitivity for and correlation with in vitro mammalian genotoxicity was low with scores of respectively 27% (21/78) and 50% (62/124). The specificity on the other hand was high with a score of 89% (41/46). Overall it can be concluded that the Vitotox™ assay is a very valuable tool for the early screening process. The test shows a high correlation with the Ames test for many structural classes. However, for a few classes it was reported that the correlation with the Ames was poor. Therefore the Ames test cannot be omitted in a later phase of the development.

The correlation of the RadarScreen assay with bacterial mutagenicity (Ames) was only 54% (78/144). Subsequently, the sensitivity and specificity of the RadarScreen for the comparison with Ames results were also low with scores of, respectively, 55% (27/49) and 54% (51/95). These results show that the RadarScreen assay cannot be used to predict the Ames results. For in vitro mammalian genotoxicity however, a correlation of 76% (93/123) was obtained. The RadarScreen assay detected direct-acting genotoxins (i.e. 4-nitroquinoline-N-oxide, cisplatin), topoisomerase inhibitors (i.e. doxorubicin), cross-linking agents (i.e. cisplatin), nucleotide/DNA synthesis inhibitors (i.e. 5-fluorouracil), and reactive oxygen species generators (i.e. hydrogen peroxide). These results indicate that the RadarScreen assay can detect compounds with different modes of action. Also in-house compounds with often an unknown mode of action and from different structural classes were detected in this assay. The three aneugenic compounds tested, i.e. taxol, colchicine, and noscapine HCl were not detected in the RadarScreen assay. Therefore it

can be concluded that this test cannot be used to detect compounds with an aneugenic mode of action. However, the application of this test in the lead optimization phase will result in compounds of higher quality in the pre-clinical phase. The regulatory genotoxicity tests in the pre-clinical phase will detect compounds with an aneugenic mode of action. Reporter assays that can (also) detect compounds with an aneugenic mode of action in the early phase of drug development would be very useful in this aspect and are currently under development in our lab.

Like in the regulatory in vitro mammalian genotoxicity assays, many of the tested steroids produced a positive result in the RadarScreen assay. In comparison with the reported data from the regulatory in vitro mammalian genotoxicity assays even more steroids showed a positive result in the RadarScreen assay. This indicates the high sensitivity of the RadarScreen assay for this compound class. Of the 12 false positive compounds in the RadarScreen assay, seven compounds were steroidal compounds. The mechanism(s) that cause(s) a clastogenic or aneugenic result for non-catechol steroids has not yet been elucidated. Therefore, more mechanistic studies are needed.

Overall it has been shown that the Vitotox™ assay predicts Ames results and the RadarScreen assay in vitro mammalian genotoxicity. The specificity of the Vitotox™ and RadarScreen assay were high for respectively Ames results and in vitro mammalian genotoxicity. The low number of false positive results is also found when all compounds are selected that were negative in both the Ames and in vitro mammalian genotoxicity test. Of these 33 compounds 22 have a negative score in both the Vitotox™ and RadarScreen assay (67%). Two aspects that can cause false positive results are cytotoxicity and precipitation. Therefore it was decided to discard measurements showing precipitation or more than 80% cytotoxicity. However, the data showed that not using these rules wouldn't have changed the number of positive/negative scores. One of the reasons might be that precipitation of compounds decreases the luminescent signal. Thus inductions that occur at a precipitating concentration will be difficult to measure in the present system.

Implementation of the Vitotox™ assay in the research phase of Organon in 2003 already affected the number of positive results in the Ames test (GLP test). Prior to the implementation of the Vitotox™ test 19% of the compounds were positive in the Ames test. After the implementation of the Vitotox™ assay and deselection of compounds with a positive result in the Vitotox™ assay Ames positive compounds were eliminated. Implementation of the RadarScreen is expected to reduce the number of compounds that are positive in in vitro mammalian genotoxicity assay.

Comparison of the RadarScreen assay with other reporter assay

The GreenScreen RAD54 assay was also tested at our lab. Although this assay, as described in various studies, responded well to known genotoxic reference compounds ,

the autofluorescence of a large number of in-house compounds interfered with the GFP measurement [10]. Surprisingly many compounds became fluorescent after incubation with yeast cells, possibly through metabolism. It has been described that the problem of autofluorescence can be avoided successfully by using fluorescence polarization (FP) [17]. However, in the case of our in-house compounds this method did not work properly. This was one of the reasons that the RadarScreen assay was introduced and validated. With luminescence as measurement technique the problems with autofluorescence were avoided. A second advantage of the highly sensitive luminescence read-out is that S9 mixture can be used to test metabolic activation of compounds in a high throughput set-up. In the GreenScreen RAD54 assay in yeast and GreenScreen HC assay, S9 mixture can be used but only in a non-HTS format [18, 19]. In comparison to the Vitotox™ assay a relatively high concentration of S9 mixture was needed in the RadarScreen assay (2%). However, in this assay compounds like benzo[a]pyrene and cyclophosphamide showed the expected positive response in the presence of S9 mixture.

The disadvantage of the assays used in the present study may be the use of non-human cells and a non-human activation system. Recently a GADD45a-GFP assay in the human lymphoblastoid cell line TK6 (GreenScreen HC, Gentronix) was developed by Hastwell et al. [18]. Their validation study showed that this assay had a high sensitivity and specificity of, respectively, 71% and 100% for in vivo genotoxicity. With a different compound set Olaharski et al. [20] showed that the sensitivity of this assay dropped to only 30% (comparison with in vitro mammalian genotoxicity). However, the specificity was 97% which makes this assay still useful for deselecting compounds in the early research phase. In response to this study Walmsley et al. send a letter to the editor [21]. In their letter they reanalyzed the data. Given the fact that in vitro genotoxicity tests give often false positive results, a carcinogen with positive in vitro mammalian genotoxicity data might still be a non-genotoxic carcinogen. They tried to make a better classification of genotoxic carcinogens and non-genotoxic carcinogens. In their analysis the sensitivity of the GADD45 assay increased to 75%. However, the compound set became smaller as it is very difficult to classify a lot of compounds. Moreover, as stated by Olaharski et al. such an analysis of the data might also introduce subjectivity. However, the importance of the comments made by Walmsley et al. surely is that in vitro mammalian genotoxicity tests give often false positive results and that this does not always reflect the classification of genotoxic/non-genotoxic carcinogenicity. Therefore it might indeed be better to compare data to the in vivo genotoxicity tests when a prediction for carcinogenicity is made. This comparison was not made in this chapter but is shown in Chapter 7.

In the present study, RadarScreen data were compared with in vitro mammalian genotoxicity data. As the yeast line used in the present study lacks features like phase II metabolism which are present in the in vivo situation, this test is expected to give more positive scores than the in vivo genotoxicity test. However, testing of the ECVAM list

showed that the number of false positive scores might be on the lower side. On the other hand, results from especially the steroidal compounds suggest the opposite.

A disadvantage of the GADD45 reporter assay is the use of GFP which can interfere with the autofluorescence of compounds. Furthermore, the cell line that is used has not been reported to contain endogenous metabolism which makes the use of a rat S9 mixture necessary which is a problem in combination with a GFP readout. It may therefore be useful to validate reporter assays with human HepG2 cells, which have endogenous metabolism [22, 23]. Such reporter cell lines which have phase II metabolism and a mammalian DNA repair system are expected to have a better correlation with in vivo genotoxicity. These cell lines are currently being developed in our lab.

Conclusion

In conclusion, the Vitotox™ assay has a good predictivity for Ames test results and the RadarScreen for in vitro mammalian genotoxicity. Implementation of the Vitotox™ and RadarScreen assays in the early research phase of drug development may be useful as it can lead to a rapid deselection of genotoxic compounds.

Conflict of interest

Annick Lauwers and Gerard Griffioen are employees of reMYND NV which develops and sells the RadarScreen assay.

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Chapter 3

Cytochrome P450 enzyme levels in HepG2 cells and cryopreserved primary human hepatocytes and their induction in HepG2 cells

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Abstract

Early in vitro toxicity screening might improve the success rate of new chemical entities in pharmaceutical development. In previous studies, the advantage of cytotoxicity screening with the HepG2 cell line was shown. Cytotoxicity could be identified for 70% of the compounds in these assays as compared with known toxicity in either in vitro assays in primary hepatocytes, in in vivo assays in rats, or in (pre-)clinical development in humans. The low phase I and II enzyme levels in HepG2 cells might have been responsible for the fact that 30% of the compounds scored negative. Therefore, we performed two follow-up studies in which cytochrome P450 (CYP) enzymes and phase II metabolism were examined.

In the present study, the transcript levels of CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4 were measured with quantitative PCR. Results showed that transcripts of all CYPs were present in HepG2 cells, however, mRNA levels of most CYPs were dramatically lower than in primary human hepatocytes. These results were confirmed with luminometric assays which were used to measure the enzyme activities of CYP1A1, 1A2, 2C9, and 3A4.

Regulation of CYP1A1, 1A2, 2B6, 2C8, 2D6, 2E1, and 3A4 by the aryl hydrocarbon receptor (AhR), pregnane X receptor (PXR) and constitutive androstane receptor (CAR) was studied in HepG2 cells at the mRNA and/or enzyme level. Regulation of CYP1A1, 1A2, 2B6, and 3A4 mRNA levels was similar to the regulation in primary human hepatocytes. In contrast, CYP2C8 mRNA levels are inducible in primary human hepatocytes, but not in HepG2 cells, after treatment with PXR/CAR activators. Consistent with other studies, CYP2D6 and 2E1 transcript levels were not changed after treatment with AhR, PXR, and CAR activators. Moreover, CYP1A1 and 1A2 enzyme levels could be induced by AhR agonists and CYP3A4 by PXR agonists.

As a consequence of the low levels of CYPs in HepG2 cells, cytotoxicity of several compounds might have been missed or underestimated as compared with cytotoxicity in primary human hepatocytes. Inducing HepG2 cells with particular receptor stimulators might lead to higher toxicity for several of the tested compounds. Compared to primary human hepatocytes, HepG2 cells are a relatively easy-to-handle tool to study the up-regulation of CYP1A1, 1A2, 2B6, and 3A4.

Introduction

Approximately 40% of the new drug candidates fail in the developmental phase due to toxicological side effects [1]. Screening on toxicity and deselection in an early phase of development of drugs may improve the success rate of new chemical entities [2]. However, this strategy implies a large number of compounds for which only a small amount of material is available. Therefore medium or high-throughput screening methods are necessary.

In two previous studies [3, 4] the advantage of early toxicity medium-throughput screening was shown with seven different fluorometric assays on four different cell lines. In these studies cytotoxicity could be shown for 70% of the compounds with known toxicity in either in vitro assays in primary hepatocytes, in in vivo assays in rats, or in (pre-)clinical development in humans. Toxicity of the remaining 30% of compounds could not be established. The human hepatoma cell line HepG2 was chosen for further toxicity screening as detoxication and activation processes of compounds are studied most optimally in liver cells. However, a discrepancy exists between Cytochrome P450 (CYP) and phase II metabolism of primary hepatocytes and HepG2 cells. Low CYP and phase II enzyme levels in HepG2 cells might have been responsible for the fact that 30% of the compounds were falsely classified as non-toxic [5-7]. However, a recent study with Cellulomics techniques on HepG2 cells counteracts the hypothesis that the metabolic competence of HepG2 cells is significantly limiting the production of reactive metabolites [8]. With this novel method, cytotoxicity in HepG2 cells could be established for more than 90% of 243 drugs with varying degrees of toxicity including drugs that produce their toxicities by a reactive metabolite. Nevertheless we performed two follow-up studies in which CYP enzymes and phase II metabolism were examined. In the present study the focus is on CYPs.

The role of phase I enzymes is to introduce a new functional group (i.e. hydroxylation) or modify an existing functional group (i.e. O-dealkylation) so as to facilitate phase II conjugation reactions. Whilst phase I reactions generally result in a more polar metabolite, it is the conjugation reactions (e.g. glucuronidation, sulfonation) that result in marked increases in water solubility and facilitate excretion.

This transformation in two steps facilitates excretion and detoxicates a wide variety of xenobiotics. For example the phase II enzyme glutathione S-transferase P1-1 prevents 4-nitroquinoline 1-oxide (4-NQO) DNA-adduct formation by the formation of 4-NQO glutathione conjugates [9,10]. However, many xenobiotics are also toxicated by CYPs and phase II enzymes [11]. A well known example is the carcinogenicity of benzo[a] pyrene in which the activities of CYP1A1 and epoxide hydrolase lead to the formation of the carcinogenic metabolite benzo[a]pyrene-7,8-diol-9,10-epoxide [12,13].

The CYP and phase II enzymes are mainly regulated by ligand-activated nuclear transcription factors, such as the aryl hydrocarbon receptor (AhR; for the CYP1A family), the constitutive androstane receptor (CAR; for the CYP2B family) and the pregnane X receptor (PXR; for the CYP3A family) [14-17].

In the present study we characterized CYP metabolism in HepG2 cells. Transcript levels and enzyme activities in the HepG2 cell line were compared with levels and activities in cryopreserved primary human hepatocytes. Quantitative PCR was used to measure mRNA levels of CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4. Enzyme activities of CYP1A1, 1A2, 2C9, and 3A4 were measured using luminometric P450-Glo substrates [18]. Furthermore, the inducibility of CYPs in HepG2 cells was studied after exposure of HepG2 cells to agonists of the xenobiotic receptors AhR, CAR and PXR. Indigo, indirubin, β-naphthoflavone (BNF), 3-methylcholanthrene (3MC), and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) were used as AhR agonists [19-21] Tularik T0901317, CITCO, rifampicin (RIF), and phenobarbital (PB) were used as PXR and/or CAR agonists. T0901317 is in principle a potent LXR ligand that is also capable of activating human PXR [22]. CITCO and RIF are potent CAR and PXR activators, respectively [23]. PB is an activator of the PXR and although it does not appear to bind directly to CAR, it is also an activator of CAR [24].

A parallel study deals with phase II metabolism and compared the mRNA levels and activities of several phase II enzymes in HepG2 cells with those of cryopreserved primary human hepatocytes [25]. Moreover, induction of phase II enzymes was studied.

Materials and methods

Materials

All compounds and reagents were of analytical grade. Indirubin was obtained from BIOMOL International (Exeter, UK), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) from Promochem (Wesel, Germany), Tularik T0901317 from NV Organon (Oss, The Netherlands), and P450-Glo substrates for CYP1A1, 1A2, 2C9, and 3A4 from Promega (Madison, USA). All other compounds were obtained from Sigma-Aldrich (St. Louis, Missouri, USA).

Cell culture

HepG2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured in Dulbecco's Modified Eagles medium and Nutrient mixture F-12 (In Vitro Technologies Inc., Baltimore, USA) mixed in a ratio of 1:1 with 10% defined supplemented bovine calf serum (dBCS) from Hyclone (Utah, USA)

and 1% Penicillin-Streptomycin (10,000 U/ml, Gibco). Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37 °C and medium was refreshed every three or four days with subculturing. All experiments in HepG2 cells were performed between passage 8 and 16. During this period, no significant change in the CYP expression was observed. For the comparison of the mRNA expression levels in HepG2 with primary hepatocytes, cells from passage 8 and 9 were used. For induction studies in HepG2, cells from passage 12, 14, and 16 were used.

Cryopreserved primary human hepatocytes

Vials containing $5x10^6$ cryopreserved primary human hepatocytes were purchased from In Vitro Technologies. After thawing the viability of the hepatocytes was assessed by using trypan blue exclusion. The viability of all vials used was >80%. Measured enzyme activities were corrected for viability. Hepatocytes from two donors were used: EG, 68 years, white female (did not use tobacco, alcohol or other substances; medical history of hypertension); HRU, 55 years, white male (did not use tobacco, alcohol or other substances; medical history of seizures and hypertension). Furthermore, 10-donor pooled cryopreserved hepatocytes with lot number KDN were used. At In Vitro Technologies the CYP and phase II enzyme activities in hepatocytes were routinely checked after thawing and hepatocytes were only released when activities were comparable to activities in fresh primary hepatocytes. Appropriate substrates were used to check the CYP1A2, 2A6, 2C9, 2C19, 2D6, 2E1, 3A4, sulfotransferase, and UDP-glucuronosyltransferase activity.

Preparation of compound solutions

Compounds were dissolved in 100% dimethyl sulfoxide (DMSO). From the stock, 10-fold dilution series were prepared in DMSO. DMSO solutions were added to culture medium leading to a final DMSO concentration of 0.1%.

RNA isolation and cDNA synthesis

HepG2 cells were seeded on petri-dishes with a density of 4500 cells/cm^2 in culture medium with 10% dBCS. After 24 h this medium was replaced with medium containing different concentrations of the inducers 3MC, BNF, indirubin, indigo, TCDD, CITCO, RIF, PB, T0901317, or vehicle alone (0.1% DMSO). After exposure for 24 h, total cellular RNA was isolated using Trizol reagent according to the manufacturer's protocol (Invitrogen, Karlsruhe, Germany).

Cryopreserved primary hepatocytes were not cultured but were directly used for RNA isolation. A vial containing $5x10^6$ hepatocytes was thawed and cells were added to 10 ml culture medium. Next cells were collected by centrifugation for 5 min at 272 g. The supernatant was removed and total cellular RNA was isolated with Trizol reagent.

For cDNA synthesis 2.5 μg total RNA from HepG2 cells or primary hepatocytes was used and 0.5 μg random hexamer primer (GE Healthcare Bio-Sciences Corp, Piscataway, USA) was added. The mixture was heated at 70°C for 10 min and thereafter quickly chilled on ice for 2 min. cDNA was synthesized in a total volume of 25 μl containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM dNTPs and 200 U Superscript II Rnase H^- Reverse Transcriptase (Invitrogen). This mixture was incubated for 1 h at 42°C. Undiluted cDNA was used for the analysis of CYP2A6, 2C9 and 2C19 expression in HepG2 cells. For all other measurements cDNA was diluted to a concentration equivalent of 10 ng/μl RNA.

Quantitative PCR

Quantitative PCR was performed using an ABI PRISM 7900 HT Sequence Detection system (Applied Biosystems, Inc. Foster City, USA). Specific primers were designed using Primer Express software (version 2.0, Applied Biosystems). To avoid the influence of DNA contamination, primer pairs were designed to span an intron-exon boundary (Table 1). Quantitative PCR was performed using cDNA equivalent to 50 ng of total RNA in a volume of 25 μl. Due to the low expression in HepG2 cells, a cDNA concentration equivalent to 500 ng of total RNA was used for analysis of CYP2A6, 2C9, and 2C19. The total mixture contained cDNA, 300 nM forward primer, 300 nM reverse primer and 1X SYBRgreen PCR Master Mix (Applied Biosystems). The program used was 10 min at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C 100%, followed by a dissociation curve step. Expression levels were normalized by using the β-actin housekeeping gene.

Specificity of luminometric P450-Glo substrates

The luminometric P450-Glo substrates are derivates of D-luciferin and converted by CYP enzymes into D-luciferin [18]. The specificity of Luciferin(Luc)-CEE, Luc-ME, Luc-H, and Luc-BE for CYP enzymes, was assessed for CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4. Firstly, a NADPH regenerating system was prepared in phosphate buffered saline (pH 7.4). This mixture contained 2.6 mM $NADP^+$, 6.6 mM glucose-6-phosphate, 6.6 mM $MgCl_2$ and 0.8 Units per ml glucose-6-phosphate dehydrogenase. The cofactor solution was prewarmed at 37ºC for 10 min. Supersomes (BD Biosciences, USA) expressing one CYP were added to this cofactor mixture. The final concentration of supersomes in the cofactor mixture was 10 pmol/ml. The cofactor/supersome mixture was kept at 37ºC. Subsequently, 50 μl of either 60 μM Luc-CEE, 200 μM Luc-Me, 200 μM Luc-H, or 200 μM Luc-BE in PBS was added to a well of a white 96-well plate (PerkinElmer, Groningen, The Netherlands). The plates with substrates were pre-heated at 37ºC for 5 min. Next, 50 μl from the cofactor/supersome mixture was added to one of the substrates. Substrates were incubated with the enzymes for 30 min and the luminescence was measured on a luminometer (TopCountNT, PerkinElmer).

Table 1. Primers used for the analysis of gene expression by quantitative PCR.

Gene	Accession number	Forward primer	Reverse primer
CYP1A1	ENST00000140465	CGGCCCCGGCTCTCT	CGGAAGGTCTCCAGGATGAA
CYP1A2	NM_000761	AGCTTCTCCTGGCCTCTGC	GGACTTTTCAGGCCTTTGGG
CYP2A6	ENST00000301141	CTATGGCTTCTTTGCTGCTCATG	CTTGCCGATCACTCTGTCAATC
CYP2B6	ENST00000324071	TTAGGGAAGCGGATTTGTCTTG	GGAGGATGGTGGTGAAGAAGAG
CYP2C8	ENST00000285985	CACCCAGAGGTCACAGCTAAAGT	CATGTGGCTCCTATCCTGCAT
CYP2C9	ENST00000260682	ACATCAGCAAATCCTTAACCAATCT	GGGTTTCAGGCCAAAATACAGA
CYP2C19	ENST00000285981	TGCTCTCCTTCTCCTGCTGAAG	TGCCAACGACACGTTCAATC
CYP2D6	ENST00000320777	CGCATCCCTAAGGGAACGA	TTCCAGACGGCCTCATCCT
CYP2E1	ENST00000252945	GCAAGAGATGCCCTACATGGA	GGGCACGAGGGGTGATGAA
CYP3A4	NM_017460	CAGGAGGAAATTGATGCAGTTTT	GTCAAGATACTCCATCTGTAGCACAGT
β-Actin	NM_001101	CTGGCACCCAGCACAATG	GCCGATCCACACGGAGTACT

Comparison of CYP1A1, 1A2, 2C9 and 3A4 enzyme activities in primary human hepatocytes and HepG2 cells with P450-Glo substrates

The P450-Glo assays were performed according to the manufacturer's protocol (Promega). Hepatocytes or HepG2 cells ($5x10^4$) were washed with PBS and resuspended in 50 µl PBS. Next 50 µl of either 60 µM Luc-CEE (CYP1A1), 200 µM Luc-ME (CYP1A2), 200 µM Luc-H (CYP2C9), or 200 µM Luc-BE (CYP3A4) was added. In each assay the substrate was present at a concentration equal to the Km value (Promega). HepG2 cells and primary hepatocytes were incubated with P450-Glo substrates for up to 1 h. Under these test conditions, substrate conversion remained linear in time with all enzymes. Due to the aspecificity of Luc-ME, CYP1A2 activity was always determined in the presence of 10^{-6} M sulphaphenazole. This concentration blocks the CYP2C9 activity completely and the CYP2C8 activity for 60% in human supersomes (data not shown). In some experiments CYP activity was blocked by coincubation with inhibitors to show the specificity of the P450-Glo substrates. CYP1A1 activity was blocked with 10^{-6} M ketoconazole, CYP1A2 activity with 10^{-6} M furafylline, CYP2C9 activity with 10^{-6} M sulphaphenazole, and CYP3A4 activity with 10^{-6} M ketoconazole. Subsequently, 50 µl luciferin detection reagent was added. Plates were shaken for 10 min and the luminescence signal was measured on a TopCountNT luminometer. The conversion rate expressed as the increase in luminescence per minute per 10^5 cells (Δluminescence/min/10^5 cells) was calculated in the linear part of the reaction time. For calculation of the amount of D-luciferin formed, a D-luciferin standard curve was made. Furthermore, cell lysates were made and the protein content of HepG2 cells and primary human hepatocytes was measured with the BCA protein assay kit from Pierce (Pierce Biotechnologies, Rockford, USA) [25].

Measurement of induction of CYP1A1, 1A2 and 3A4 enzyme activities in HepG2 cells

HepG2 cells were trypsinized, counted and resuspended in culture medium to a final concentration of $3x10^4$ cells/well for the CYP1A1 and CYP1A2 assay and to $1.5x10^5$ cells/well for the CYP3A4 assay. 190 µl cell suspension was added to a well of a white 96-well culture plate (Perkin Elmer). The 96-well microtiter plates were incubated for 24 h in a humidified atmosphere at 37°C under 5% CO_2. Next day 10 µl medium containing different concentrations of 3MC, BNF, indirubin, indigo, TCDD, CITCO, RIF, PB, T0901317, or vehicle alone (0.1% DMSO) was added. Subsequently, plates were incubated for another 24 h in a humidified atmosphere at 37°C under 5% CO_2. After exposure, plates were washed twice with PBS and the CYP1A1, 1A2, and 3A4 activities were assessed by using 50 µl Luc-CEE (30 µM), Luc-ME (100 µM), or Luc-BE (100 µM). In each assay the substrate was present at a concentration equal to the Km value. Under these test conditions, substrate conversion remained linear in time with all

enzymes. Next 50 µl luciferin detection reagent was added and plates were shaken for 10 min. Then the luminescence signal was measured with a TopCountNT luminometer. For calculation of the amount of D-luciferin formed, a D-luciferin standard curve was made.

Statistical analysis

Each experiment was performed in duplicate in three independent experiments except where indicated otherwise. Data are expressed as mean ± SEM. Data obtained from mRNA and enzyme activity induction studies were compared for statistically significant differences using the unpaired Student's *t* test ($p<0.05$). For the mRNA induction studies, only induction levels above 2-fold with statistically significant differences were seen as relevant.

Results

Comparison of CYP mRNA expression in HepG2 cells and primary human hepatocytes

Transcripts of CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4 were present in both cryopreserved hepatocytes and HepG2 cells, although mRNA levels of most CYP enzymes were much lower in HepG2 cells (Figure 1). CYP1A1 levels were 3- and 37-fold higher in hepatocytes from donor EG and HRU. In contrast CYP1A1 levels in the donor pool were 10-times lower than those in HepG2 cells. Levels of the second CYP1A family member, CYP1A2, were 24-, 386-, and 55-fold higher in hepatocytes from donor EG, HRU, and the pool. Likewise, Cyp2B6 transcript levels were 10-fold higher in the donor pool and more than 100-fold higher in donor EG and HRU. Expression levels of CYP2D6 differed less than 100-fold between the hepatocytes and HepG2 cells. Levels of CYP2D6 were 92-, 44-, and 7-fold higher in hepatocytes from donor EG, HRU, and the pool. Furthermore, levels of CYP2A6, 2C8, 2C9, 2C19, 2E1, and 3A4 were generally between 100- and 1000-fold higher in the hepatocytes.

Specificity of P450-Glo substrates

The specificity of the luminometric substrates was assessed for 11 human CYP isoforms (Figure 2). Luc-CEE showed the greatest specificity for CYP1A1 and showed very little reactivity with the other CYPs. The second luminometric substrate Luc-ME showed the greatest reactivity with CYP1A2 as expected. However, this substrate also reacted substantially with CYP2C8 and 2C9. CYP2C8 and 2C9 reactivity was 24% and 32% of the reactivity with CYP1A2.

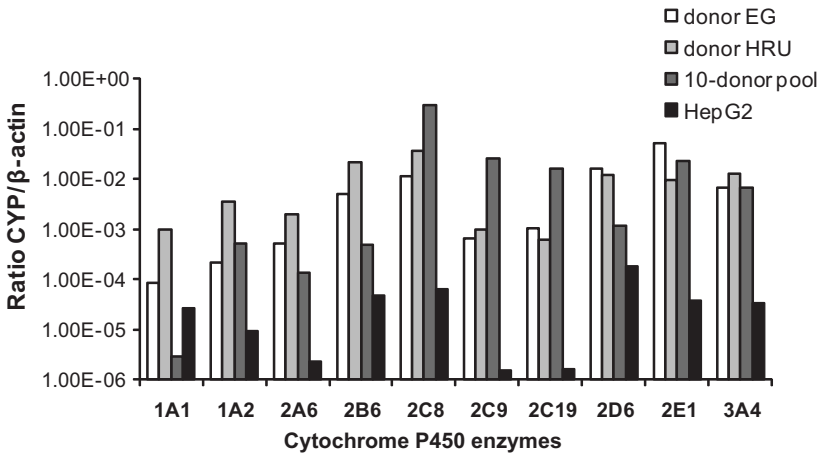

Figure 1. Cytochrome P450 (CYP) 1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4 mRNA expression levels in cryopreserved primary human hepatocytes (donor EG, donor HRU, 10-donor pool) and HepG2 cells. Results are presented as the mean mRNA levels expressed in copy number ratio of the CYP and β-actin transcripts of two independent measurements.

Thus to assess CYP1A2 activity, CYP2C8 and 2C9 were inhibited with 10^{-6} M sulphaphenazole. This concentration blocked CYP2C9 completely and 60% of CYP2C8 activity but had no effect on CYP1A2 activity (data not shown). Luc-H was very specific and reacted only with CYP2C9. Luc-BE was quite specific for CYP3A4 and showed only little reactivity with CYP1A1 (16%) and 2C8 (14%).

Comparison of CYP enzyme activities in HepG2 cells and primary human hepatocytes

The CYP activities in HepG2 cells and donor EG expressed in Δluminescence/min/10^5 cells are shown in Figure 3. Results were in concordance with the mRNA levels measured. CYP1A1 activity was higher in donor EG as compared with activity in HepG2 cells and could be inhibited with 10^{-6} M ketoconazole. CYP1A2 activity was detectable in both primary hepatocytes and HepG2 cells, but was 80-times lower in HepG2 cells. The CYP1A2 activity could be blocked completely with 10^{-6} M furafylline. The activity of CYP2C9 was high in donor EG but not detectable in HepG2 cells. CYP2C9 activity in the hepatocytes could be blocked completely with 10^{-6} M sulphaphenazole. Furthermore, results showed that CYP3A4 activity in HepG2 cells was detectable, but 15-times lower than the activity in donor EG. Activities could be inhibited substantially with 10^{-6} M ketoconazole. A D-luciferin standard curve showed that an increase of 10,000 light units is equal to the formation of 0.62 pmol D-luciferin (data not shown). Moreover, the protein content of 10^5 HepG2 cells and hepatocytes was 0.28 and 0.16 mg, respectively.

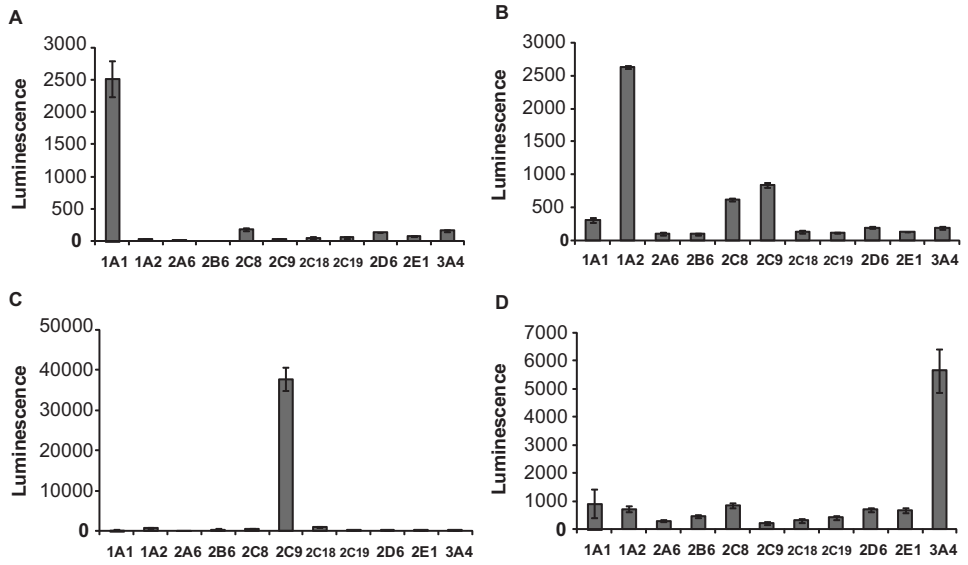

Figure 2. Specificity of Luciferin(Luc)-CEE (A), Luc-ME (B), Luc-H (C), and Luc-BE (D) for the human cytochrome P450 isotypes 1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, and 3A4. Results are presented as the mean activity of 3 independent measurements ± SEM.

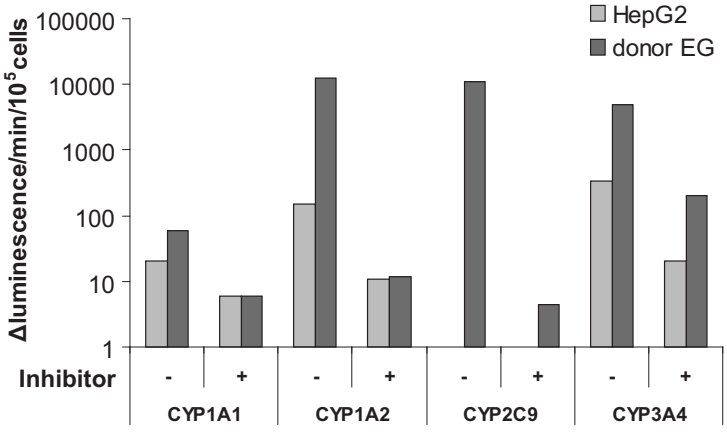

Figure 3. Cytochrome P450 (CYP) 1A1, 1A2, 2C9, and 3A4 activity (Δluminescence/min/10^5 cells) in cryopreserved primary human hepatocytes (donor EG) and HepG2 cells. CYP1A1, 1A2, 2C9, and 3A4 enzyme activities were blocked with 10^{-6} M ketoconazole, furafylline, sulphaphenazole, and ketoconazole, respectively. Results are presented as the mean activity of two independent measurements (-, basal activity; +, activity with inhibitor).

Effects of AhR activators on CYP mRNA expression

The transcript levels of CYP1A1, 1A2, 2B6, 2C8, 2D6, 2E1, and 3A4 were measured after 24 h exposure to AhR agonists. The very low expression and consequently the large amount of cDNA needed made it impossible to study the up-regulation of CYP2A6, 2C9, and 2C19. None of the AhR agonists changed CYP2C8, 2D6 and 2E1 transcript levels (data not shown). The effects on the transcript levels of CYP1A1, 1A2, 2B6, and 3A4 are presented in Figure 4.

As expected, transcript levels of CYP1A1 were highly induced after exposure to the five AhR agonists. The lowest activation doses for BNF, indirubin, indigo, TCDD, and 3MC were 10^{-7} M, 10^{-8} M, 10^{-8} M, 10^{-10} M, and 10^{-8} M, respectively. The highest activation doses for BNF, indirubin, indigo, TCDD and 3MC (with fold changes in parentheses) were 10^{-4} M (239), 10^{-4} M (1359), 10^{-4} M (85), 10^{-7} M (452), and 10^{-5} M (487), respectively.

Likewise, CYP1A2 was induced after exposure to the five AhR agonists. The lowest activation doses for BNF, indirubin, indigo, TCDD and 3MC were 10^{-7} M, 10^{-8} M, 10^{-8} M, 10^{-10} M, and 10^{-8} M. The highest activation doses for BNF, indirubin, indigo, TCDD and 3MC (with fold changes in parentheses) were 10^{-4} M (51), 10^{-4} M (244), 10^{-4} M (444), 10^{-7} M (487), and 10^{-5} M (276), respectively.

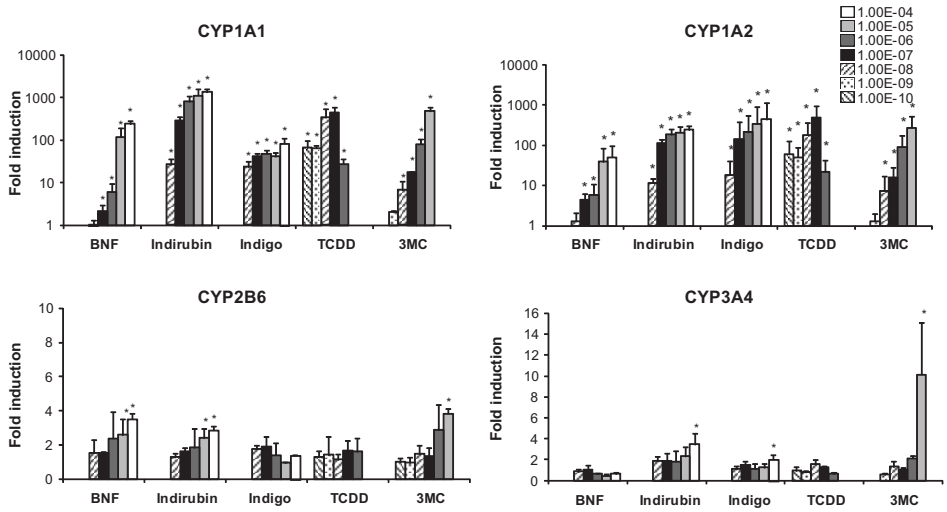

Figure 4. Induction of cytochrome P450 (CYP) 1A1, 1A2, 2B6, and 3A4 mRNA expression in HepG2 cells after 24 h treatment with the AhR activators β-naphthoflavone (BNF), indirubin, indigo, TCDD, and 3-methylcholanthrene (3MC). The level in uninduced HepG2 cells is set at 1. Data are presented as the mean expression ± SEM (n=3). *Significant difference ($p<0.05$)

Surprisingly, CYP2B6 and CYP3A4 transcript levels were also enhanced after treatment with some of the AhR agonists, but fold changes were 10- to 100-fold lower than for the CYP1A isoforms.

CYP2B6 transcript levels were induced after treatment with BNF, indirubin and 3MC. However, indigo and TCDD treatment did not change the CYP2B6 mRNA levels. The lowest activation dose for BNF, indirubin, and 3MC was 10^{-5} M. The maximum induction of 3- to 4-fold was observed at 10^{-4} M for BNF and indirubin, and at 10^{-5} M for 3MC.

CYP3A4 was induced after exposure to indirubin, indigo and 3MC. No effect of BNF and TCDD was observed. After exposure to 10^{-4} M indirubin and indigo an induction of 3.5- and 2-fold was measured. A higher induction of 10-fold was observed at 10^{-5} M 3MC.

Effects of PXR and CAR activators on CYP mRNA expression

The expression levels of CYP enzymes in HepG2 cells were measured after 24 h exposure to several PXR/CAR agonists. Transcript levels of CYP2C8, 2D6, and 2E1 were not enhanced by the CAR/PXR agonists (data not shown). CYP1A1, 1A2, 2B6, and 3A4 mRNA levels were significantly induced by one or more of the tested activators (Figure 5).

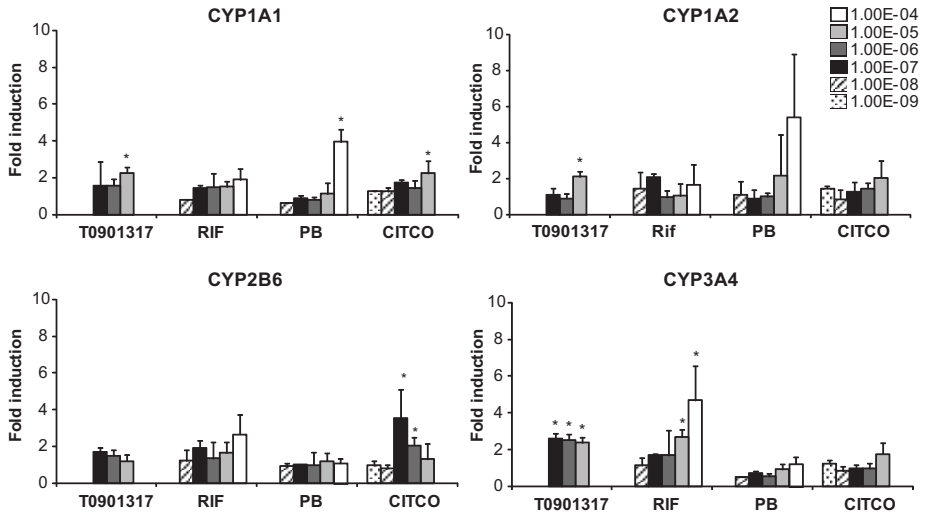

Figure 5. Induction of cytochrome P450 (CYP) 1A1, 1A2, 2B6, and 3A4 mRNA expression in HepG2 after 24 h treatment with the PXR/LXR activator T0901317, PXR activator rifampicin (RIF), the CAR/PXR activator phenobarbital (PB), and the CAR activator CITCO. The level in uninduced HepG2 cells is set at 1. Data are presented as the mean expression ± SEM (n=3). *Significant difference ($p<0.05$)

CYP1A1 mRNA levels were enhanced after exposure to 10^{-5} M T0901317 and CITCO, and 10^{-4} M PB. However, induction was only very small (2- to 4-fold) in comparison to inductions of between 100- and more than 1000-fold observed after treatment with the AhR agonists. CYP1A2 transcript levels were only slightly increased after exposure to 10^{-5} M T0901317.

CYP2B6 was induced 3.5-fold after treatment with 10^{-7} M CITCO. No effect of T0901317, RIF, and PB on CYP2B6 expression was observed.

CYP3A4 transcript levels were induced after treatment with T0901317 and RIF. No effects were seen with PB and CITCO. The lowest activation doses for T0901317 and RIF were 10^{-7} M and 10^{-5} M. The highest activation doses for T0901317 and CITCO were 10^{-7} M and 10^{-4} M, leading to 2.6- and 4.7-fold induction, respectively.

Effects of activators on enzyme activities of CYP1A1, 1A2, and 3A4

The CYP1A1 and 1A2 activities were assessed with Luc-CEE and Luc-ME for BNF, indirubin, indigo, TCDD, and 3MC. The induction of CYP1A1 and 1A2 enzyme activities by AhR agonists are shown in Figure 6. The AhR agonists tested induced the CYP1A1 enzyme activities above the basal CYP1A1 activity in HepG2 cells. TCDD was the most potent CYP1A1 inducer with a maximum induction of almost 1000-fold at 10^{-8} M and a significant induction of almost 10-fold at 10^{-11} M. Indirubin, indigo and 3MC were also potent inducers (>100-fold). Indirubin, indigo and 3MC induced CYP1A1 activity at concentrations as low as 10^{-8} M. BNF was the weakest inducer of CYP1A1 (10-fold).

Moreover, the five AhR agonists induced CYP1A2 enzyme activities above the basal CYP1A2 activity. TCDD induced CYP1A2 activity at concentrations as low as 10^{-10} M and at 10^{-9} M the maximum induction was 15-fold. Indirubin enhanced CYP1A2 enzyme activity at all tested concentrations, induction being observed at 10^{-8} M and the maximal induction of 25-fold was observed at 10^{-6} M. BNF, indigo and 3MC were weaker inducers of the CYP1A2 enzyme activity than TCDD and indirubin. These compounds showed a maximum induction of between 5- and almost 10-fold. The lowest activation dose for BNF and indigo was 10^{-6} M, while for 3MC it was 10^{-7} M.

CYP3A4 activity was assessed with Luc-BE for T0901317 and PB. RIF could not be tested because the dark red color of this compound disturbs the luminescence measurement. Treatment with T0901713 induced CYP3A4 activity significantly above the basal activity (Figure 7). A maximum induction just above 2-fold was measured at 10^{-5} M. No effect of PB on CYP3A4 expression was observed.

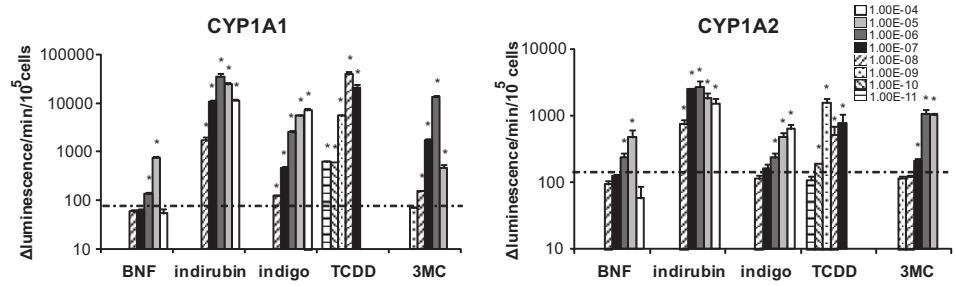

Figure 6. Dose dependent induction of cytochrome P450 (CYP) 1A1 and 1A2 enzyme activities (Δluminescence/min/10^5 cells) after 24 h treatment with β-naphthoflavone (BNF), indirubin, indigo, TCDD, and 3-methylcholanthrene (3MC). The dashed lines indicate the basal enzyme activities in HepG2 cells. Data are presented as the mean activity ± SEM (n=3).

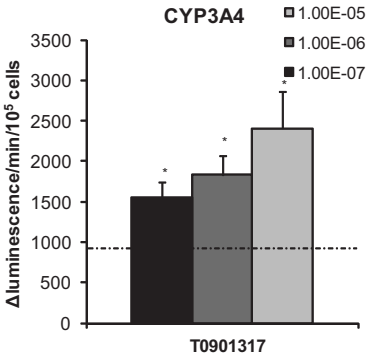

Figure 7. Dose dependent induction of cytochrome P450 (CYP) 3A4 activity (Δluminescence/min/10^5 cells) after 24 h treatment with T0901317. The dashed line indicates the basal CYP3A4 activity in HepG2 cells. Data are presented as the mean activity ± SEM (n=3).

Discussion

In the present study the CYP mRNA expression and enzyme activities in HepG2 cells and cryopreserved primary human hepatocytes were compared. The HepG2 cell line is derived from a Caucasian human hepatoblastoma and has both phase I and II metabolizing enzymes [5, 26, 27]. Hewitt and Hewitt [5] showed that the enzyme activities depend on the source and culture conditions and that consequently characterization of HepG2 cells is essential.

Results from the present study showed that the transcript levels and enzyme activities of most CYPs were much lower in HepG2 cells as compared to levels in primary human hepatocytes. The mRNA levels of CYP1A1, 1A2, 2B6, 2C8, 2C9, and 2C19 in the three

primary hepatocyte sources showed a variation of more than 10-fold. This might have been due to genotype or food intake.

CYPs play a pivotal role in the toxication of some compounds [28, 29]. Thus the low enzyme levels in HepG2 cells might result in an underestimation of the toxicity of compounds as compared with toxicity in primary human hepatocytes. On the other hand, cytotoxicity was observed in HepG2 for several compounds like iproniazid, dacarbazine, nitrofurantoin, and benzo[a]pyrene that need metabolic activation via CYP3A4, 1A1, and/or 1A2 [3, 4]. Similar observations were made by O'Brien et al. [8]. This implies that for several compounds, the CYP activities in HepG2 cells are high enough or that the compounds themselves activate a xenobiotic receptor, which induces CYP expression.

Rodriguez et al. [6] showed that the low CYP expression in HepG2 and other permanent hepatoma cells is caused by a decreased CYP transcription. A change in hepatic transcription factors might be involved in this process. Support for this hypothesis comes from transfection studies with C/EBPα in HepG2 cells, leading to higher levels of CYP2B6, 2C9, and 2D6 [30]. Another way to induce CYP enzyme levels is by treatment with AhR, CAR, and PXR agonists.

CYP1A1 contributes to the toxicity of many carcinogens, especially PAHs [31, 32, 33]. The main pathway for CYP1A1 induction is through activation of the AhR pathway [34]. In the present study all five AhR activators used highly induced CYP1A1 mRNA levels and enzyme activities. TCDD was the most potent inducer followed by indirubin and indigo. Indirubin and indigo were identified in human urine in normal donors and can be endogenous ligands of the AhR as both compounds are present in the human serum at a concentration of 10^{-8} M [19]. In yeast AhR activation assays, these compounds were comparable or even more potent than TCDD [35]. Remarkably also the PXR/CAR activators PB, CITCO, and T0901317 induced CYP1A1 transcript levels, although to much lower levels than real AhR agonists. CYP1A1 and/or CYP1A2 induction by PXR/CAR activators has been observed in other studies as well [36, 37].

CYP1A2 activates PAHs, nitrosamines and aryl amines into DNA-binding forms [38]. CYP1A2 induction on transcriptional level can occur through AhR dependent and independent pathways [39] or through post-transcriptional pathways [40]. In the present investigation all five AhR activators induced the CYP1A2 transcript levels by 10- to 100-fold, which was only slightly weaker than observed for CYP1A1. Of the PXR/CAR activators only T0901317 showed statistically significant induction. Before induction, CYP1A2 levels were between 24- and 386-fold lower. The induction leads to comparable or even higher mRNA and enzyme levels than in cryopreserved hepatocytes.

CYP2B6 metabolizes endogenous substances such as testosterone [41], serotonin [42], and xenobiotics of which clinical drugs such as cyclophosphamide [43] and bupropion [44] are examples. In the present study, CYP2B6 was induced by the specific CAR agonist CITCO, while RIF showed only a tendency for induction. Regulation of

CYP2B6 by these ligands via CAR and PXR has also been documented by others [15, 45, 46]. T0901317 and PB did not induce CYP2B6 levels although CYP2B6 induction by PB was described in primary human hepatocytes by others [45, 47]. Madan et al. (2003) [45] showed a large variation in CYP2B6 induction from 1.3-fold induction to a 17-fold induction in 13 different donors. Surprisingly, the AhR agonists BNF, indirubin, and 3MC also induced CYP2B6 expression in HepG2 cells. The concentrations needed for CYP2B6 activation were higher than those for CYP1A1 and CYP1A2. Classical AhR ligands and atypical CYP1A inducers were recently profiled in rats by microarray experiments [48]. These authors also found that some of the AhR activators including flutamide, omeprazole and indole-3-carbinol showed cross-talk at high concentrations with PXR/CAR pathways leading to CYP2B and CYP3A induction. Similar data were found for CYP2B6 in human hepatocytes with benzo[a]pyrene [7]. These findings suggest that at high concentrations, some AhR agonists give cross-talk with the CAR/PXR pathway. Enzyme levels of CYP2B6 have not yet been measured since a proper specific fluorophore or luminophore is still unavailable.

CYP3A4 metabolizes approximately 50% of the drugs that are currently on the market and plays an important role in the toxication of compounds [29]. Examples are CYP3A4 metabolites of flutamide, troglitazone, and isoniazid which cause hepatotoxicity [49-51]. CYP3A4 is regulated by CAR and PXR [52-54]. In this study CYP3A4 mRNA regulation was shown with the PXR agonists RIF and T0901317, the latter being more potent. CYP3A4 was not induced by the CAR agonists CITCO and PB. It has recently been observed in human hepatocytes that although PXR and CAR both regulate CYP3A4 and CYP2B6 like in rodents, human CAR preferentially induces CYP2B6 relative to CYP3A4 because of its weak binding and activation of CYP3A4 ER6 [47]. In the study of Faucette et al. [47] treatment of human hepatocytes with 0.5-1 μM CITCO resulted in a more than 2-fold mRNA induction of CYP3A4 in only one out of six donors. Cross-talk of the AhR agonists indirubin, indigo and 3MC with the PXR/CAR pathways leading to induction of CYP3A4 was also demonstrated in the present study.

The CYP2C subfamily contains four members of which CYP2C8, CYP2C9, and CYP2C19 are expressed in the liver [55]. In the present study, regulation of CYP2C8 was studied. CYP2C8 plays a role in the oxidative metabolism of taxol, arachidonic acid, and retinoids [56]. In human hepatocytes CYP2C8 is inducible with PXR/CAR activators such as CITCO, dexamethasone, RIF, and PB [57, 58]. None of the AhR, PXR, and CAR activators used in the present study changed the transcript levels of CYP2C8. These results are not consistent with findings in primary human hepatocytes.

Consistent with other reports, CYP2D6 was a non-inducible enzyme [37, 59]. Regulation of CYP2E1 can occur by exposure to xenobiotics, such as alcohol, isoniazid, and pyridine [60-63]. However, as for CYP2D6, no involvement of the AhR, PXR, CAR or other nuclear receptors in the regulation of CYP2E1 has been observed in this study

or by others.

In conclusion, HepG2 cells have low levels of CYPs. The results regarding CYP1A1, 1A2, 2B6, and 3A4 induction show that there is a good correlation with earlier results observed in primary human hepatocytes. Furthermore, CYP2D6 and CYP2E1 could, like in primary hepatocytes, not be induced with AhR, PXR, and CAR agonists. In contrast to results observed in primary human hepatocytes no regulation of CYP2C8 with PXR/CAR activators was found in HepG2 cells. Still, HepG2 cells are an easy to handle tool in contradiction to primary hepatocytes to study the regulation of CYP1A1, 1A2, 2B6, and 3A4. Prestimulating HepG2 cells with AhR, PXR, and CAR activators before performing cytotoxicity assays might lead to a better predictivity of toxicity.

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Chapter 4

Phase II enzyme levels in HepG2 cells and cryopreserved primary human hepatocytes and their induction in HepG2 cells

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Abstract

The HepG2 cell line is a valuable tool for screening for cytotoxicity in the early phase of pharmaceutical development. Some compounds which produce reactive and toxic metabolites, are classified as being toxic in HepG2 cells. In contrast, other compounds, which are toxic in primary human hepatocytes, are not toxic in HepG2 cells. A difference in metabolism between HepG2 cells and primary human hepatocytes might be the reason. To investigate this, cytochrome P450 and phase II enzyme levels were characterized. In the present study the focus is on phase II enzyme metabolism.

Transcript levels of UDP-glucuronosyl transferases (UGTs), sulfotransferases (SULTs), glutathione S-transferases (GSTs), N-acetyltransferase-1 (NAT1) and epoxide hydrolase (EPHX1) were measured with quantitative PCR in HepG2 cells and cryopreserved primary human hepatocytes. Levels of SULT1A1, 1A2, 1E1, 1A2, and 2A1, microsomal GST 1, GST μ1, NAT1, and EPHX1 in HepG2 cells were almost similar to levels in primary human hepatocytes. In contrast, levels of UGT1A1 and 1A6 transcripts were between 10- and more than 1000-fold higher in the primary hepatocytes.

The regulatory processes of phase II enzymes by the aryl hydrocarbon receptor, pregnane X receptor and constitutive androstane receptor were studied in HepG2 cells and appeared quite similar to those in primary human hepatocytes.

Due to the involvement of phase II enzymes in the toxication of some compounds, HepG2 cells can be a valuable cellular system to predict toxicity for these compounds. On the other hand, the normal expression of most phase II enzymes in combination with the lower expression of cytochrome P450 enzymes in HepG2 cells might result in an underestimation of toxicity for several compounds. Compared to primary human hepatocytes, HepG2 cells are a relatively easy-to-handle tool to study the up-regulation of phase II enzymes.

Introduction

For screening purposes in the early phase of pharmaceutical development, the HepG2 cell line can be useful [1-4]. For instance, tamoxifen, benzo[a]pyrene, and nitrofurantoin which need metabolic activation in order to express their toxicity, show cytotoxicity in HepG2 cells [2-5]. In contrast, some compounds reported as being toxic in primary human hepatocytes, are classified as non-toxic in HepG2 cells. A difference in metabolism between HepG2 cells and primary human hepatocytes might cause this discrepancy [6-8]. Therefore we performed two studies in which either cytochrome P450 (CYP) or phase II metabolism in HepG2 cells was characterized. In the present study the focus is on phase II metabolism.

In phase II metabolism a polar group in a compound is used for the conjugation reaction with an endogenous hydrophilic compound. The phase II reactions generally lead to more polar molecules and facilitate biliary and renal excretion. The major phase II metabolizing enzymes are the UDP-glucuronosyltransferases (UGTs), sulfotransferases (SULTs), glutathione S-transferases (GSTs), arylamine N-acetyltransferases (NATs), and epoxide hydrolases (EPHXs). The products of phase II reactions are often less toxic than the parent compound or products of phase I reactions. An example of a detoxication reaction is the detoxication of 4-nitroquinoline 1-oxide (4NQO). 4NQO induces DNA adduct formation and is detoxicated by the formation of 4NQO-glutathione conjugates [9, 10].

However, phase II biotransformations can also result in enhanced toxicities. A few examples are the role of epoxide hydrolase in the activation of benzo[a]pyrene [11], the conversion of the pesticide ethylene dibromide to a half-mustard/episulfonium ion by glutathione transferases [12], and the modification of aryl hydroxylamines and benzylic alcohols by sulfonylation which leads to the formation of reactive nitrenium ions and carbocations respectively [13].

Some polymorphisms in phase II enzymes are linked to higher risks towards side effects of drugs [14]. A polymorphism in NAT2, leading to reduced enzyme activity, is proposed to lead to a higher risk for side effects of isoniazid treatment [15]. Furthermore, slow acetylator phenotypes are associated with a weaker induction of the micronucleus frequency after treatment with radioactive iodine and fast acetylators are associated with a higher risk for colorectal cancer [16, 17]. Polymorphisms in UGT1A1 may lead to mild or severe hyperbilirubinemia, i.e. Gilbert's syndrome and Crigler-Najjar syndrome [18]. Phase II enzymes are mainly regulated by the aryl hydrocarbon receptor (AhR), the constitutive androstane receptor (CAR), and pregnane X receptor (PXR) [19-22].

In the present study we characterized phase II metabolism in HepG2 cells. Transcript levels and enzyme activities in the HepG2 cell line were compared with those in cryopreserved primary human hepatocytes. Quantitative PCR was used to measure

transcript levels of UGT1A1, 1A6, SULT1A1, 1A2, 1E1, 2A1, microsomal GST 1 (mGST-1), GST µ1, NAT1, and EPHX1. Monochlorobimane and 4-methylumbelliferone were used to measure the overall GST enzyme activity [23, 24] and the overall UGT activity [25], respectively. In addition, the induction of phase II enzymes in HepG2 cells was determined after exposure to several AhR, CAR, and PXR agonists.

Materials and methods

Materials

All compounds and reagents were of analytical grade. Most compounds, as well as monochlorobimane (MCB), 4-methylumbelliferone (4MU) and uridine diphosphate glucuronic acid (UDPGA) were obtained from Sigma-Aldrich (St. Louis, Missouri, USA). Indirubin was obtained from BIOMOL International (Exeter, UK), T0901317 from NV Organon (Oss, The Netherlands), and 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) from Promochem (Wesel, Germany).

Cell culture

HepG2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured in Dulbecco's Modified Eagles medium and Nutrient mixture F-12 (Invitrogen, Kalsruhe, Germany) mixed in a ratio of 1:1 with 10% defined supplemented bovine calf serum (dBCS) from Hyclone (Utah, USA) and 1% Penicillin-Streptomycin (10,000 U/ml, Invitrogen). Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37 °C and medium was refreshed every 3 or 4 days with subculturing. All experiments in HepG2 cells were performed between passage 8 and 16. During this period, no significant change in the phase II enzyme expression was observed (data not shown). For the comparison of the mRNA expression levels in HepG2 with primary hepatocytes, cells from passage 8 and 9 were used. For induction studies in HepG2, cells from passage 12, 14, and 16 were used.

Cryopreserved primary hepatocytes

Vials containing $5x10^6$ cryopreserved primary human hepatocytes were purchased from In Vitro Technologies (Baltimore, USA). After thawing, the viability of the hepatocytes was assessed by using trypan blue exclusion. The viability of all vials used was above 80%. The measured enzyme activities were corrected for viability. Hepatocytes from two donors were used: EG, white female (68 years, did not use tobacco, alcohol or other substances; medical history of hypertension); HRU, white male (55 years, did not use tobacco, alcohol or other substances; medical history of seizures and hypertension).

Furthermore, 10-donor pooled cryopreserved hepatocytes with lot number KDN were used. At In Vitro Technologies the CYP and phase II enzyme activities in hepatocytes were routinely checked after thawing and hepatocytes were only released when activities were comparable to activities in fresh primary hepatocytes. Appropriate substrates were used to check the CYP1A2, 2A6, 2C9, 2C19, 2D6, 2E1, 3A4, SULT, and UGT activities.

Preparation of compound solutions

Compounds were dissolved in 100% dimethyl sulfoxide (DMSO). From the stock, 10-fold dilution series were prepared in DMSO. DMSO solutions were added to culture medium leading to a final DMSO concentration of 0.1%.

Compound incubation, RNA isolation and cDNA synthesis

Cryopreserved primary hepatocytes were not cultured but were directly used for RNA isolation. A vial containing $5x10^6$ hepatocytes was thawed and cells were added to 10 ml culture medium. Next cells were collected by centrifugation for 5 min at 272 *g*. The supernatant was removed and total cellular RNA was isolated with Trizol reagent according to the manufacturer's protocol (Invitrogen).

HepG2 cells were seeded on petri-dishes with a density of 4500 cells/cm^2 in culture medium with 10% dBCS. After 24 h this medium was replaced by medium containing 0.1% DMSO or a dose range of various AhR, PXR, and CAR agonists. 3-methylcholanthrene (3MC), β-naphthoflavone (BNF), indirubin, indigo, and TCDD were used as AhR agonists [26-28]. Rifampicin (RIF) and Tularik T0901317 were used as PXR agonists [29], CITCO as CAR agonist [30] and phenobarbital (PB) as an agonist of the PXR and CAR receptor [31]. The cytotoxicity of the activators was tested in the Calcein-AM and glutathione depletion assays as described previously [3, 4]. Of the activators used 3MC and TCDD showed a cytotoxic effect at $3.16x10^{-5}$ and $1.00x10^{-6}$ M, respectively. 3MC was in the induction studies used up to a non-toxic maximum concentration of $1x10^{-5}$ M. The highest concentration TCDD (10^{-6} M) used in the induction studies, showed a cytotoxic effect (40% inhibition).

After an incubation period with the activators of 24 h, RNA was isolated using Trizol reagent. For cDNA synthesis 2.5 μg total RNA from HepG2 cells or primary human hepatocytes was used and 0.5 μg random hexamer primer (GE Healthcare Bio-Sciences Corp, Piscataway, USA) was added. The mixture was heated at 70°C for 10 min and thereafter quickly chilled on ice for 2 min. cDNA was synthesized in a total volume of 25 μl containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM dNTPs and 200 U Superscript II Rnase H^- Reverse Transcriptase (Invitrogen). After incubation for 1 h at 42°C, cDNA was diluted to a concentration equivalent of 10 ng/μl RNA.

Quantitative PCR

Quantitative PCR was performed using an ABI PRISM 7900 HT Sequence Detection system (Applied Biosystems, Inc. Foster City, USA). Specific primers were designed using Primer Express software (version 2.0, Applied Biosystems). To avoid the influence of DNA contamination, primer pairs were designed to span an intron-exon boundary, except for NAT1 which is an intronless gene [32] (Table 1).

Quantitative PCR was performed using cDNA equivalent to 50 ng RNA in a total of 25 µl PCR mix. The total mixture contained cDNA, 300 nM forward primer, 300 nM reverse primer and 1X SYBRgreen PCR Master Mix (Applied Biosystems). The program used was 10 min at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C (100% ramp), followed by a dissociation curve step. Expression levels were normalized by using the β-actin housekeeping gene.

Preparation of cell lysates for enzyme analysis

HepG2 cells ($3x10^7$) were trypsinized and added to 10 ml culture medium. A vial containing $5x10^6$ primary human hepatocytes was thawed and added to 10 ml culture medium. Next HepG2 cells and primary human hepatocytes were centrifuged for 10 min at 300 *g*. Subsequently, cells were resuspended in 600 µl cold 0.25 M sucrose solution, buffered with 0.01 M Tris-HCl (pH 7.4), and chilled on ice. A cell suspension of 200 µl was lysed by three subsequent sonifications with a sonifier (Branson Ultrasonics Corporation, Danbury, USA) at 4 °C for 20 s with a 40 s interval between the pulses.

UGT activity measurements with 4-methylumbelliferone

The overall UGT activity was determined by using 4-MU as substrate. The reaction mixture contained 4-MU (50 µM), the cofactor uridine diphosphate glucuronic acid (UDPGA, 1mM), and cell lysate in a final volume of 100 µl.

The amount of protein in the reaction mixture was 0.55-0.64 mg and 0.04-0.08 mg for HepG2 cells and human hepatocytes, respectively. Substrate and lysate were preincubated for 2 min at 37ºC. The reaction was started by addition of UDPGA and followed for 20 min at 37ºC. The decrease in fluorescent signal was measured each minute on the Victor II (Perkin Elmer, Groningen, The Netherlands). The excitation wave length was set at 340 nm and the emission was measured at 460 nm. The conversion rate was calculated in the linear part of the reaction curve. For calculation of the amount of 4-MU converted, a 4-MU standard curve was made.

Table 1. Primers used for the analysis of gene expression by quantitative PCR.

Gene	Accession number	Forward primer	Reverse primer
UGT1A1	ENST00000305208	AACTTTCTGTGCGACGTGGTT	GTCACCTCTCTCTGAAGGAATTCTG
UGT1A6	AY435141	AGCCCAGACCCTGTGTCCTA	CCACTCGTTGGGAAAAAGTCA
SULT1A1	ENST00000305384	GTCACCGAGCTCCCATCTTC	GTCTCCATCCCTGAGGGAATC
SULT1A2	ENST00000319640	CCCCAGACTCTGTTGGATCAG	AACCGCCACATCCTTTGC
SULT1E1	ENST00000226444	TGGTGGCTGGTCATCCAAA	GAACCTGTCCTTGCATGAATTTC
SULT2A1	ENST00000222002	TCCAGTTATTCCCCAAGTCTTTCT	AAACATCTCTGGGATTTCTCATGAG
mGST-1	ENST00000320716	GCAGAGCCCACCTGAATGAC	CGGGACCACTCAAGGAATACAG
GSTμ1	ENST00000234981	TTTAGGCCTGTCTGCGGAAT	CCAGCCCGCGGATGT
NAT1	ENST00000307719	TGGGAGGATTGCATTCAGTCT	TGCTTCTTTCCTGGCTTGAATTC
EPXHX1	ENST00000339714	TGCACATCCAGTGCACCAA	CCAGACCCACAGGAGAGTCATT
β-Actin	NM_001101	CTGGCACCCAGCACAATG	GCCGATCCACACGGAGTACT

GST activity measurements with monochlorobimane

The overall GST enzyme activity was assayed fluorometrically with monochlorobimane (MCB). The reaction mixture contained 40 µM MCB, 1 mM reduced glutathione (GSH), and cell lysate in a total volume of 100 µl. The amount of protein in the reaction mixture was 0.12-0.15 mg and 0.04-0.08 mg for HepG2 cells and human hepatocytes, respectively. The reaction was started by the addition of GSH. Conjugation of MCB with glutathione results in formation of the fluorescent product Gs-bimane which was measured at 37°C on the Victor II for 10 min every 20 s. The excitation wave length was set at 355 nm and the emission was measured at 460 nm. The conversion rate was calculated in the linear part of the reaction curve. The glutathione adduct of MCB (Gs-bimane) is not commercially available. Therefore the method described by Bai et al. [33] was used for the calculation of the amount of Gs-bimane formed. Under optimized reaction circumstances (surplus enzyme and cofactor) several amounts of MCB (10-50 µM with steps of 5 µM) were completely converted into Gs-bimane. The resulting standard curve was used for the calculation of the amount Gs-bimane formed.

Protein quantification

Protein concentration of cell lysates was measured with the BCA protein assay kit from Pierce (Pierce Biotechnologies, Rockford, USA) which is based on bicinchoninic acid (BCA) for the colorimetric detection and quantification of total protein. The assay was performed according to the manufacturer's protocol.

Statistical analysis

Each experiment was performed in duplicate in three independent experiments except where indicated otherwise. Data are expressed as mean ± SEM. The obtained data were compared for statistically significant differences using the unpaired Student's *t* test ($p<0.05$). Only induction levels above 2-fold with statistically significant differences were seen as relevant.

Results

Comparison of phase II enzyme expression between HepG2 cells and primary human hepatocytes

The transcripts of UGT1A1 and 1A6, SULT1A1, 1A2, 1E1, and 2A1, mGST-1, GST µ1, NAT1 and EPHX1 were present in both HepG2 cells and hepatocytes (Figure 1). With exception of UGTs, the mRNA levels in HepG2 cells differed only slightly from levels in cryopreserved human hepatocytes.

Transcript levels of UGT1A1 were dramatically lower in HepG2 cells as compared to cryopreserved hepatocytes. The levels were 235-, 838-, and 163-fold higher in donor EG, HRU, and the pool, respectively. Likewise, UGT1A6 mRNA expression was 397-, 2749- and 14-fold higher in donor EG, HRU, and the pool, respectively.

SULT1A1 and 1A2 mRNA expression was almost similar in HepG2 cells and the hepatocytes (<3-fold difference). Transcript levels of SULT1E1 were 4- and 2-fold higher in donor HRU and the donor pool respectively and 13-fold lower in donor EG. The levels of the fourth SULT isotype analyzed, SULT2A1, were 3-fold higher in donor EG and the donor pool. Substantially higher levels (32-fold) were measured in donor HRU.

Transcript levels of the GST isotypes, mGST-1 and GST µ1, were only slightly different from levels in HepG2 cells. mGST-1 mRNA expression in donor EG, HRU, and the pool was 14- and 20-fold higher or 7-fold lower, respectively. The difference between expression of GST µ1 in hepatocytes and HepG2 cells was even less. Expression was 5-fold higher, almost equal, and 14-fold lower in donor EG, HRU, and the pool, respectively.

NAT1 transcript levels were almost equal in donor HRU, slightly higher in donor EG (5-fold), and slightly lower in the donor pool (4-fold).

EPHX1 mRNA levels were almost equal in HepG2 and the donor pool. Somewhat greater differences were observed between HepG2 cells and donor EG and HRU in which transcript levels were 6- and 34-fold higher, respectively.

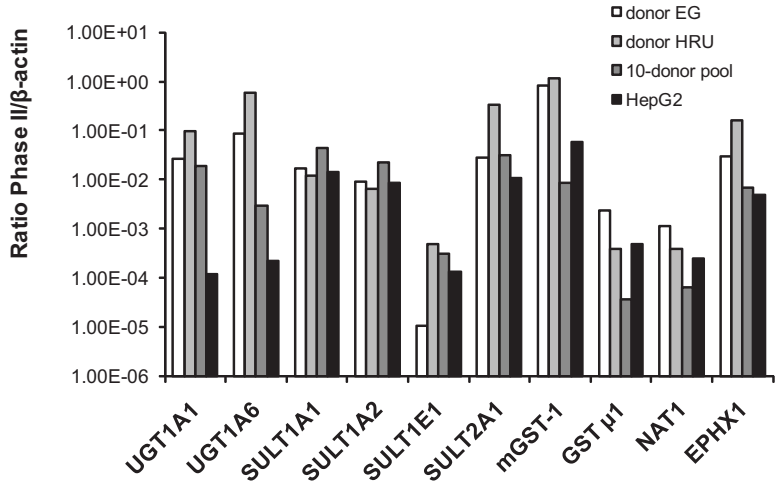

Figure 1. UDP-glucuronosyltransferases (UGT1A1 and 1A6), sulfotransferases (SULT1A1, 1A2, 1E1, and 2A1), glutathione S-transferases (mGST-1, GST µ1), N-acetyltransferase 1 (NAT1), and epoxide hydrolase 1 (EPHX1) expression levels in cryopreserved primary human hepatocytes (donor EG, donor HRU, 10-donor pool) and HepG2 cells. Results are presented as the mean mRNA levels expressed in copy number ratio of the phase II enzyme and β-actin transcripts of two independent measurements.

Comparison of phase II enzyme activities in HepG2 cells and primary human hepatocytes

The overall UGT and GST activities were detectable in both HepG2 cells and the donor pool, but activities were significantly higher in the donor pool (Figure 2).

The low transcript levels in HepG2 cells of UGT1A1 and 1A6 are associated with a lower UGT enzyme activity (47-fold). The GST activity in HepG2 cells differed only slightly with the activity in the donor pool.

Although expression levels of the GST isotypes mGST-1 and GST μ1 were around 10-fold higher in HepG2 cells as compared with the donor pool, the overall GST enzyme activity was 4-fold lower in HepG2 cells.

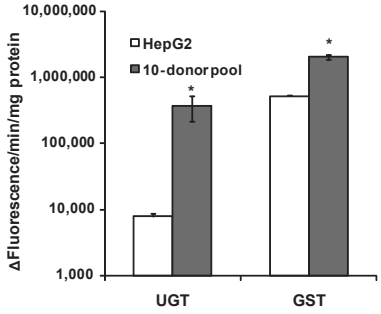

Figure 2. UDP-glucuronosyl (UGT) and glutathione S-transferase (GST) activity (Δfluorescence/min/mg protein) in cryopreserved primary human hepatocytes and HepG2 cells. Results are presented as the mean activity ± SEM (n=3). A Δfluorescence/min of 100,000 is equal to the conversion of 0.33 nmol/min 4-MU and formation of 0.89 nmol/min GS-bimane. *Significant difference ($p<0.05$)

Effects of AhR activators on phase II enzyme expression in HepG2 cells

The effects of several AhR agonists on the transcript levels of the UGT- and SULT-isoforms analyzed are presented in Figure 3.

Transcript levels of UGT1A1 were markedly increased after exposure to the AhR agonists. Exposure to the inducers resulted in a dose-dependent induction of UGT1A1 expression. The lowest activation doses for BNF, indirubin, indigo, TCDD, and 3MC were 10^{-7}, 10^{-8}, 10^{-7}, 10^{-10}, and 10^{-8} M, respectively. The highest activation doses for BNF, indirubin, indigo, TCDD, and 3MC (with fold changes in parentheses) were 10^{-4} (8), 10^{-5} (20), 10^{-5} (6), 10^{-8} (6), and 10^{-5} M (22). Likewise, exposure to the AhR inducers resulted in increased UGT1A6 transcript levels. The lowest activation doses for BNF, indirubin, indigo, TCDD, and 3MC were 10^{-8}, 10^{-8}, 10^{-7}, 10^{-10}, and 10^{-6} M, respectively. The highest activation doses for BNF, indirubin, indigo, TCDD, and 3MC (with fold changes in parentheses) were 10^{-4} (16), 10^{-5} (19), 10^{-4} (6), 10^{-8} (23), and 10^{-5} M (5). Induction of the SULT isotypes by the AhR activators was less prominent. SULT1A1 was induced by all AhR activators, with exception of 3MC. The lowest activation doses

were 10^{-7} M for BNF and TCDD, and 10^{-5} M for indirubin, and indigo. The maximum induction of 2- to 3-fold was observed for BNF, indirubin, indigo, TCDD at 10^{-6}, 10^{-5}, 10^{-5} , and 10^{-7} M, respectively.

SULT1A2 transcript levels were induced after exposure to BNF, indirubin, indigo, and 3MC, in contrast to TCDD which did not show an effect. The induction was observed at a concentration as low as 10^{-7} M for BNF and indirubin, and at 10^{-6} M for indigo and 3MC. The maximum induction (2- to 6-fold) was observed at 10^{-5} M for indirubin, and at 10^{-6} M for BNF, indigo and 3MC.

None of the inducers changed SULT1E1 transcript levels (data not shown).

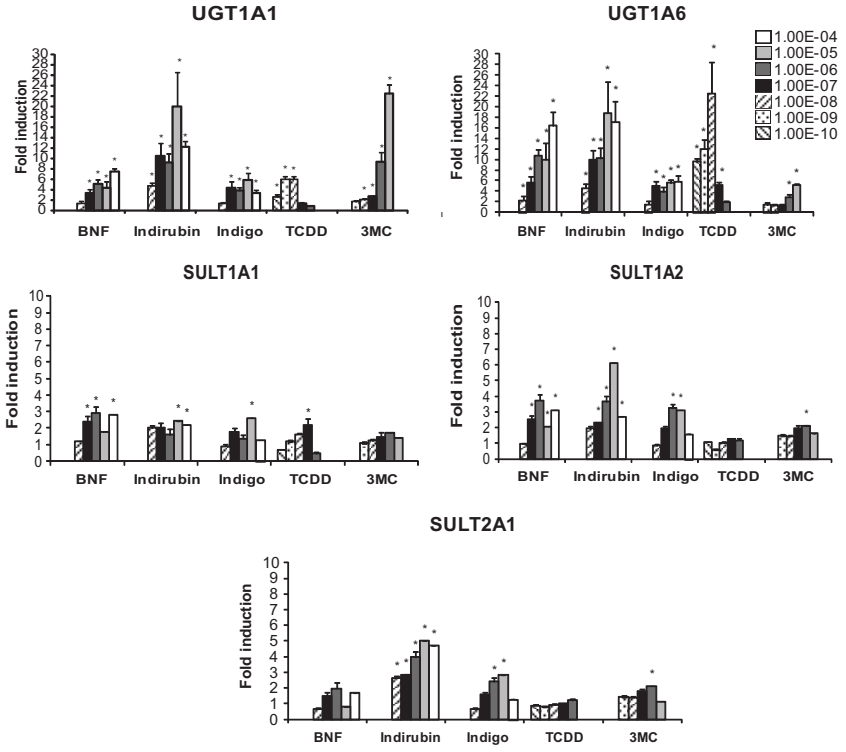

Figure 3. Induction of UDP-glucuronosyltransferase (UGT) 1A1 and 1A6, sulfotransferase (SULT) 1A1, 1A2, 1E1, and 2A1 mRNA expression in HepG2 cells after 24 h treatment with the AhR agonists β-naphthoflavone (BNF), indirubin, indigo, TCDD, and 3-methylcholanthrene (3MC). The level in uninduced HepG2 cells is set at 1. Data are presented as the mean expression ± SEM (n=3). *Significant difference ($p<0.05$)

SULT2A1 was induced by indirubin, indigo and 3MC. The lowest activation doses observed were 10^{-8} M and 10^{-6} M for indirubin and indigo, respectively. The maximum induction for both compounds was observed at 10^{-5} M, leading to 5- and 2.8-fold induction for indirubin and indigo, respectively. 3MC caused only a slight increase in SULT2A1 levels that was only significant at 10^{-6} M (2-fold). BNF and TCDD did not show an effect.

The effects of the AhR inducers on the mRNA levels of mGST-1, GST µ1, NAT1, and EPHX1 are presented in Figure 4. mGST-1 transcript levels were enhanced by BNF, indirubin and indigo. The lowest activation doses for BNF, indirubin, and indigo were 10^{-7}, 10^{-8}, and 10^{-5} M. The highest activation doses for BNF, indirubin, and indigo (with fold changes in parentheses) were 10^{-6} (5.6), 10^{-4} (3.8), and 10^{-5} M (2.7), respectively.

GST µ1 was slightly induced (2.8-fold) after treatment with 10^{-5} M indirubin. NAT1 was also only significantly induced after exposure to indirubin at 10^{-4} M, which caused a 2.5-fold induction of the mRNA expression. Induction of EPHX1 transcript levels after exposure to AhR inducers was more prominent. The five AhR agonists all caused a concentration dependent induction of EPHX1. The lowest activation doses for BNF, indirubin, indigo, TCDD, and 3MC were 10^{-6}, 10^{-6}, 10^{-5}, 10^{-9}, and 10^{-6} M, respectively. The highest activation doses for BNF, indirubin, indigo, TCDD, and 3MC were 10^{-4}, 10^{-5}, 10^{-5}, 10^{-7}, and 10^{-5} M, respectively, resulting in a 2- to 7-fold induction.

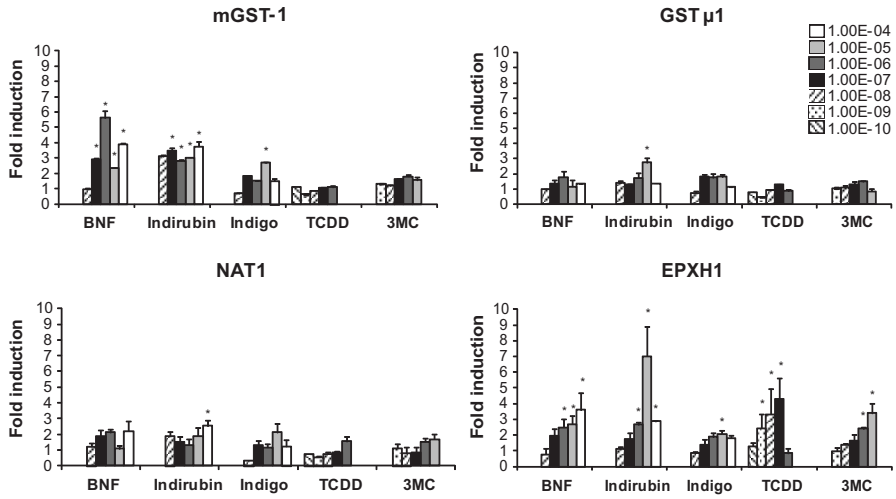

Figure 4. Induction of microsomal glutathione S-transferase 1 (mGST-1), glutathione S-transferase Mu 1 (GST µ1), N-acetyltransferase 1 (NAT1), and epoxide hydrolase 1 (EPHX1) mRNA expression in HepG2 after 24 h treatment with the AhR agonists β-naphthoflavone (BNF), indirubin, indigo, TCDD, and 3-methylcholanthrene (3MC). The level in uninduced HepG2 cells is set at 1. Data are presented as the mean expression ± SEM (n=3). *Significant difference ($p<0.05$)

Effects of PXR and CAR activators on phase II enzyme expression in HepG2 cells

The mRNA induction of phase II enzymes was assessed after 24 h exposure T0901317, RIF, PB, and CITCO (Figure 5). None of these activators changed the transcript levels of UGT1A6, SULT1A1, SULT1E1, mGST-1, GST μ1, and EPHX1 (data not shown).

Treatment with the CAR and/or PXR agonists resulted in induction of UGT1A1 expression. However, induction was less prominent than induction with the AhR agonists. A maximum induction of approximately 3-fold was measured at 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-7} M for T0901317, RIF, PB, and CITCO, respectively. Induction was already significant at concentrations as low as 10^{-7}, 10^{-6}, 10^{-8} and 10^{-9} M for T0901317, RIF, PB, and CITCO, respectively.

SULT1A2 expression was induced by RIF at a concentration as low as 10^{-7} M with a maximum induction of 2.7-fold at 10^{-5} M. The lowest effective concentration of PB was also 10^{-7} M and the maximum induction of 2.6-fold was observed at 10^{-6} M. Induction by CITCO already occurred at 10^{-8} M with a maximum induction of 3.5-fold at 10^{-7} M. In contrast T0901317 did not affect SULT1A2 mRNA expression.

CITCO induced SULT2A1 transcript levels at a concentration as low as 10^{-9} M and at 10^{-7} M the maximum induction was observed (4.4-fold). PB did not cause such a prominent induction, however, at 10^{-5} M and 10^{-6} M an induction of 2-fold was measured. No changes in transcript levels were observed after exposure to T09001317 and RIF.

NAT1 was significantly induced (2-fold) after exposure to PB and CITCO.

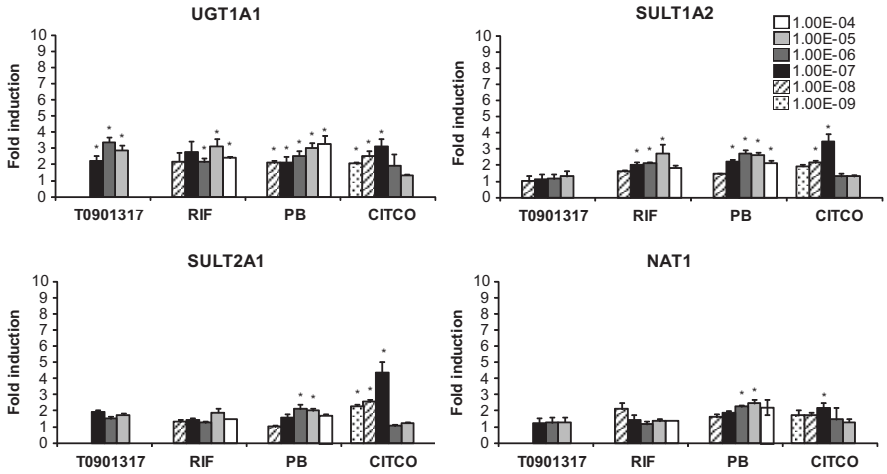

Figure 5. Induction of UDP-glucuronosyltransferase 1A1 (UGT1A1), sulfotransferase (SULT) 1A2 and SULT2A1, and N-acetyltransferase 1 (NAT1) mRNA expression in HepG2 after 24 h treatment with the PXR/LXR agonists T0901317, PXR agonist rifampicin (RIF), the CAR/PXR agonist phenobarbital (PB), and the CAR agonist CITCO. The level in uninduced HepG2 cells is set at 1. Data are presented as the mean expression ± SEM (n=3). *Significant difference ($p<0.05$)

Discussion

It was demonstrated that HepG2 cells have with exception of UGTs a complete set of phase II enzymes. Levels of SULT1A1, 1A2, 1E1, mGST-1, GST μ1, NAT1 and EPHX1 in HepG2 cells differed only modestly from those in human cryopreserved primary hepatocytes. Also the overall GST enzyme activity in HepG2 cells was almost equal to that of primary hepatocytes. On the other hand, the mRNA levels of UGT1A1 and 1A6 and the overall UGT enzyme activity were much higher in hepatocytes than in HepG2 cells. These results are consistent with findings in other studies [6, 8, 34]. The fact that UGTs from the UGT1 family are highly inducible by agonists of the AhR, PXR, and CAR [19, 35] might have been due to this difference. Ritter et al. [35] showed that the levels of UGT1A1 in freshly isolated hepatocytes decreased dramatically after three days in culture. They hypothesized that a lack of induction factors, i.e. AhR, PXR, CAR agonists, caused this drop in activity. Consistent with our findings Wilkening et al. [8] found a high mRNA expression and SULT enzyme activity. In contrast Grant et al. [37] observed a low SULT activity and a high UGT activity in HepG2 cells as compared to human hepatocytes. This stresses that characterization of HepG2 cells is important. Hewitt and Hewitt [6] found that the mRNA expression levels and activities of phase I and II enzymes in HepG2 cells are dependent on the source and culture conditions.

As shown previously, the levels of CYP enzymes in HepG2 cells are low as compared to levels in primary human hepatocytes [38]. Due to these low CYP enzyme levels and more physiological levels of most phase II enzymes there might be a balance towards the detoxication process for some of the compounds. This might explain why some compounds with known toxicity in primary hepatocytes remain non-toxic in HepG2 cells, whereas others like tamoxifen, benzo[a]pyrene and nitrofurantoin, being AhR, PXR and/or CAR inducers, remain toxic in both HepG2 cells [3, 4] and primary hepatocytes.

Phase II enzymes can also play a crucial role in the toxication of several compounds. For these compounds the high levels of most phase II enzymes might reflect the cytotoxic effects in both HepG2 cells and primary hepatocytes. For example, HepG2 cells having high NAT levels predict mutagenicity of heterocyclic aromatic amines in in vitro micronucleus tests better than cell lines with low levels of NAT [34]. However, low levels of UGTs in HepG2 cells might underestimate the toxicity of some compounds, since excessive glucuronidation leads to toxicity with clofibric acid and gemfibrozil [39, 40].

We also examined the regulation of phase II enzymes by AhR, PXR and CAR agonists. UGT1A1 was shown to be responsive to 3MC, BNF, PB and RIF in primary human hepatocytes [41]. This can be explained by the fact that AhR, PXR and CAR binding motifs are present in the promoter region of UGT1A1 [19, 35]. Consistent with these

findings, UGT1A1 was in the present study induced after treatment with all the AhR, PXR and CAR agonists used. Induction with the AhR agonists was most prominent.

UGT1A6 was also induced by treatment with the five AhR agonists, but no effects were seen with PXR and CAR agonists. This is in agreement with the study of Bock and Kohle [19]. Regulation of UGT1A6 by the AhR is also supported by the fact that UGT1A6 is not inducible by TCDD in AhR knockout mice [42].

The superfamily of sulfotransferases consists of at least ten functional genes in humans. Four members (SULT1A1, 1A2, 1E1, and 2A1) which are expressed in the liver, were studied. SULT1A1 is involved in the detoxication and bioactivation of many xenobiotics, including proximate carcinogens. SULT1A1 transcript levels were in the present study upregulated by the AhR inducers BNF, indirubin, indigo, and TCDD. A tendency of induction was shown with 3MC. Therefore, it is likely that SULT1A1 is regulated via the AhR pathway. These results are in line with a study in which treatment of HepG2 cells with BNF resulted in induction of SULT1A1 expression [43]. None of the PXR and CAR agonists affected SULT1A1 mRNA levels. Likewise, in other studies treatment of human hepatocytes with PXR/CAR inducers like PB, CITCO, dexamethasone, and RIF did not result in SULT1A1 mRNA induction [44].

In vitro studies have shown that SULT1A2 can activate proximate carcinogenic heterocyclic aromatic amines more efficiently into carcinogenic metabolites than SULT1A1. However, SULT1A2 protein has not yet been detected in vivo and data about regulation of SULT1A2 are not reported [45, 46]. Here, exposure of HepG2 cells to BNF, indirubin, indigo, and 3MC resulted in increased SULT1A2 transcript levels, however, TCDD did not show any effect. SULT1A2 expression was also induced by the PXR/CAR agonists RIF, PB, and CITCO. These results suggest that AhR, PXR and CAR might all play a role in the regulation of SULT1A2 expression.

SULT1E1, is known as an estrogen sulfotransferase and has been extensively studied due to its important role in steroid homeostasis [47, 48]**.** No induction of SULT1E1 transcript levels by AhR, PXR, or CAR agonists was shown by us and others.

The SULT2 family mainly sulfonates neutral steroids and sterols. An important member of this family is SULT2A1, which catalyzes sulfonation of a number of endogenous hydroxysteroids, bile acids, and polycyclic xenobiotics such as certain aromatic carcinogens and therefore plays an important role in activation or detoxication reactions [49, 50]. It has been shown that SULT2A1 is regulated by the farnesoid X receptor [51], vitamin D receptor [52, 53], CAR, and PXR [54]. In the present, study SULT2A1 was regulated by the CAR inducers CITCO and PB. Although induction of SULT2A1 by PXR agonists has been reported, treatment of HepG2 cells with RIF and T0901317 did not result in induction of SULT2A1 mRNA expression. Also the AhR agonists BNF and TCDD did not affect SULT2A1 expression. However, up-regulation was observed with the AhR agonists indirubin, 3MC and indigo.

Glutathione S-transferases detoxicate a large number of electrophiles [55]. Nevertheless they are also involved in the activation of some proximate carcinogens such as haloethanes and haloalkenes [56]. GSTs can be classified into cytosolic and membrane bound GSTs. The regulation of membrane bound mGST-1 and cytosolic GST μ1 which are both abundantly present in the liver were studied. Induction of membrane bound GSTs after in vivo treatment of rats with PB and BNF have been reported [57]. In the present study none of the PXR and/or CAR agonists affected mGST-1 transcript levels. However, the AhR inducers BNF, indirubin, and indigo increased mGST-1 mRNA levels. TCDD did not show an effect and, although not significant, 3MC showed a tendency of induction. These results suggest that there might be a role for AhR in mGST-1 regulation. The regulation of the different cytosolic GSTs has been studied in detail in rat and human hepatocytes [58, 59]. In these studies 3MC and PB both induced cytosolic GSTs, however, no or very small inductions of GST μ1 were observed. Consistent with this, in the present study also very slight or no effects were seen with the AhR, PXR and/or CAR inducers.

NAT1 was induced 2-fold after exposure to indirubin, CITCO, and PB. The effects of the CAR agonists CITCO and PB suggest that CAR might play a role in the regulation of NAT1. Indirubin was the only AhR agonist that affected NAT1 transcript levels. Therefore it is probably not AhR but crosstalk with another receptor that caused this effect. In another study the AhR activator benzo[a]pyrene also increased NAT1 transcript levels in HepG2 cells and primary human hepatocytes [8].

EPHX1 transcript levels were increased after treatment with the five AhR inducers tested. Regulation of EPHX1 by AhR agonists was consistent with results from previous studies in which induction of EPHX1 was shown with benzo[a]pyrene [8] and Araclor 1254 [60]. Several studies reported a slight induction (<2-fold) of EPHX1 after treatment with PB [60, 61]. Here no induction with the PXR and CAR inducers was observed.

At the highest concentration of TCDD used (10^{-6} M), 40% cytotoxicity was shown in the glutathione depletion and Calcein-AM assay. This might explain the drop in UGT1A1, UGT1A6, SULT1A1, and EPHX1 induction seen at 10^{-6} M.

In conclusion, HepG2 cells have with exception of UGTs a complete set of phase II enzymes. However, mosts conclusions are based on mRNA expression and not on enzyme activities since proper (specific) fluorometric or luminometric substrates for NAT1, SULTs, and EPHX1 are not yet available. Regulation of phase II enzymes in HepG2 cells shows that there is a good correlation with previous results in primary human hepatocytes. Therefore, in contradiction to primary hepatocytes, HepG2 cells are a relatively easy-to-handle tool for studying regulation of phase II enzymes in human liver cells.

Because of the role of these phase II enzymes in catalyzing the toxication of several compounds, HepG2 cells might become a valuable tool system to predict toxicity. On the

other hand, levels of most CYPs are low in HepG2 cells without pretreatment of AhR/PXR/CAR agonists [38]. Therefore for compounds in which CYP metabolism plays a crucial role in toxication and phase II metabolism plays a pivotal role in detoxication, toxicity might be underestimated due to a shift towards phase II metabolism. This imbalance might explain why some compounds with known toxicity in primary human hepatocytes remain non-toxic in HepG2 cells.

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Chapter 5

Development and validation of a high content screening in vitro micronucleus assay in CHO-k1 and HepG2 cells

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Abstract

In the present study an automated image analysis assisted in vitro micronucleus assay was developed using both the in regulatory genotoxicity assays commonly used rodent CHO-k1 cell line as well as the human HepG2 hepatoma cell line. The HepG2 cell line was chosen because the properties of this cell line including the presence of a functionally active p53 protein, a functionally competent DNA repair system, enzymes for phase I and II metabolism, and an active Nrf2 electrophile responsive system, might result in an assay with a high predictivity for in vivo genotoxicity.

The CHO-k1 and HepG2 based assays were both evaluated by testing a compound list recommended by the European Centre for the Validation of Alternative Methods (ECVAM) that contains in vivo genotoxins and non-genotoxins. The sensitivity (percentage of genotoxic compounds that tested positive) (80%; 16/20) and specificity (percentage of non-genotoxic compounds that tested negative) (88%; 37/42) of the CHO-k1 cell line were high. Although the sensitivity of the HepG2 cell line was lower, being 60% (12/20), the specificity was high amounting to 88% (37/42). These results were confirmed by testing an additional set of 16 genotoxic compounds. For both the CHO-k1 as well as HepG2 cell line it was possible to size-classify micronuclei enabling discrimination of aneugens from clastogens.

It is concluded that two high-throughput micronucleus assays were developed that can detect genotoxic potential and allow differentiation into clastogens and aneugens. The performance scores of both the CHO-k1 and HepG2 cell line for in vivo genotoxicity were high. Application of these assays in the early discovery phase of drug development may proof to be a useful strategy to early assess genotoxic potential.

Introduction

In drug development toxicity is an important factor for drug attrition. Genotoxicity is one of the causes of toxicity. The current regulatory in vitro genotoxicity assays used for determining genotoxic potential, have a low-throughput and need a relatively large amount of compound and are therefore in their present format less applicable in the early discovery phase. Toxicity screening in the early discovery phase, requires assays that have a high-throughput and need a low amount of compound. High-throughput assays based on the use of bacteria, yeast, and rodent/human cell lines are proposed to be useful in vitro models.

In the present study a high content screening (HCS) technique was used to develop a high-throughput in vitro micronucleus assay in the rodent Chinese Hamster Ovarian k1 (CHO-k1) cell line as well as the human HepG2 cell line. In the HCS technique (fluorescence) microscopy is used to analyze cell lines in 96-well plates (or even 384-well plates). Aspects that are of importance for development of a fast HCS micronucleus assay are automatic scoring, the choice of the cell line, the validation with proper reference compounds, and the ability of the test system to discriminate clastogens from aneugens.

Scoring of micronuclei in the regulatory in vitro micronucleus (IVMN) assay is a labor intense method that is performed manually on microscopic slides by trained operators. The manual scoring of micronuclei using microscopic slides results in inter-observer and intra-observer variability, with a scorer specific, coefficient of variation between 5.5 and 9.5% [1]. One approach to improve the efficiency is the automated counting of micronuclei by image analysis [1]. With this aim Diaz et al. [2] developed a HCS IVMN assay in CHO-k1 cells. Validation with reference compounds showed that their CHO-k1 HCS IVMN assay was a high-throughput alternative with respect to the manual scoring of micronuclei in microscopic slides. This method was in the present study further optimized with respect to speed and stability of the stains.

To assess the use of HCS in the development of an IVMN assay two cell lines were compared in the present study being the CHO-k1 and HepG2 cell line. The CHO-k1 cell line is frequently used in the regulatory IVMN assay where it showed a high sensitivity (78.7%) (percentage of carcinogenic compounds that tested positive) but a low specificity (30.8%) (percentage of non-carcinogenic compounds that tested negative) for carcinogenicity [3]. This low specificity makes the regulatory assay not very practical for toxicity screening in early discovery phase of drug development, as it may lead to inefficient deselection of pharmacologically interesting compounds. The low specificity of the CHO-k1 cell line may be due to a mutation in the p53 protein ($Thr^{211} > Lys^{211}$) that affects its functionality [4-6]. This p53 protein plays a pivotal role in cell cycle control

and activation of DNA repair and apoptosis. CHO-k1 cells do not show endogenous phase I and II metabolism, which makes the addition of a metabolic fraction (i.e. S9 mixture), necessary. Application of S9 mixture can give false positive results in comparison to in vivo genotoxicity due to excessive phase I metabolism (outside the cellular membrane) without phase II metabolism [7]. As HepG2 cells contain phase I and II metabolism, an active Nrf2 electrophile responsive system, a functionally active p53 protein, and active DNA repair, the predictivity for in vivo genotoxicity may be higher [8-12].

The HCS IVMN assays in the two cell lines were evaluated by using a list of 62 reference compounds for validation of new in vitro genotoxicity tests, published by the European Centre for the Validation of Alternative Methods (ECVAM) [13]. Based on available data, 20 compounds were defined by an ECVAM expert panel as being genotoxic and 42 compounds as non-genotoxic. This non-genotoxic group contains 19 compounds that are frequently scored as falsely positive. Besides these compounds an additional set of 16 reference compounds including several estrogenic compounds, scoring positive for genotoxicity, were tested. The sensitivity (percentage of genotoxic compounds that test positive) and specificity (percentage of non-genotoxic compounds that test negative) of the CHO-k1 and HepG2 cell line based assays were compared.

Another aspect that has to be taken into account when developing a micronucleus assay is the discrimination of clastogens from aneugens. In the conventional IVMN assay, clastogens and aneugens are discriminated by using fluorescent in situ hybridization (FISH) analysis. This method is difficult to perform in a high-throughput format. Recently Hashimoto et al. [14] have shown that size-classification of micronuclei is a reliable measure which is as effective as the commonly used FISH analysis for the discrimination of aneugens from clastogens. For the new automated image analysis based assays a HCS method to size-classify micronuclei to discriminate aneugens from clastogens was developed and incorporated in the image analysis software.

In summary, the focus in the present study is on 4 subsequent steps all required for development of a new micronucleus assay including (1) the development and optimization of an automated image analysis assisted HCS in vitro micronucleus assay in the rodent CHO-k1 cell line as well as the human HepG2 cell line, (2) the validation of these HCS IVMN assays with proper model compounds for genotoxicity, (3) the comparison of the performances of the CHO-k1 and HepG2 cell line and (4) development of a method to discriminate aneugens from clastogens based on size-classification of the micronuclei.

Materials and methods

Materials

Dulbecco's modified Eagles medium, Nutrient Mixture F-12 (DMEM/HAM F12 medium in a ratio of 1:1) was ordered from Gibco (Invitrogen, Carlsbad, CA, USA) and defined supplemented bovine calf serum was ordered from Hyclone (Thermo Scientific, Logan, Utah, USA). Both Collagen I and poly-D-lysine coated black/clear 96-wells microplates were obtained from BD Biosciences (San Jose, California, USA).

Compounds

Stock solutions of the compounds were prepared in 100% dimethyl sulfoxide (DMSO). From the stocks, √10-fold dilution series were prepared in DMSO. All compounds were of analytical grade. Most of the compounds were ordered from Sigma-Aldrich (St. Louis, Missouri, USA), except 2-amino-3-methylimidazo[4, 5-f]quinoline (IQ) which was ordered from Wako (Richmond, Virginia, USA), and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP.HCl) which was ordered from Toronto research (North York, Ontario, Canada).

Cell culture

CHO-k1 and HepG2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Both cell lines were used between passage 5 and 15. Cells were cultured in Dulbecco's Modified Eagles medium and Nutrient mixture F-12 (In Vitro Technologies Inc., Baltimore, USA) mixed in a ratio of 1:1 with 10% defined supplemented bovine calf serum from Hyclone (Utah, USA), 1% Penicillin-Streptomycin (10,000 U/ml, Gibco), and 0.05% of an aqueous mixture containing ethanolamine (2.44 ml/l), sodium selenite (0.9 mg/l) and 2-mercaptoethanol (4.2 ml/l). Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37 °C and medium was refreshed every three or four days with sub-culturing.

Preparation of assay plates

The most appropriate plate coating of 96-well plates was assessed with respect to an even distribution and stretching of cells. This is especially an issue with HepG2 cells as these cells form easily clumps and grow in layers, which results in unfocused areas during imaging.

For CHO-k1 cells collagen-I coated plates gave good results (Figure 1). However, HepG2 cells formed easily clumps and multiple layers on this coating. For HepG2 cells poly-D-lysine plates gave proper results, with only a limited number of cell clumps (Figure 1). Therefore CHO-k1 cells were seeded on collagen-I plates and HepG2 cells on poly-D-lysine plates.

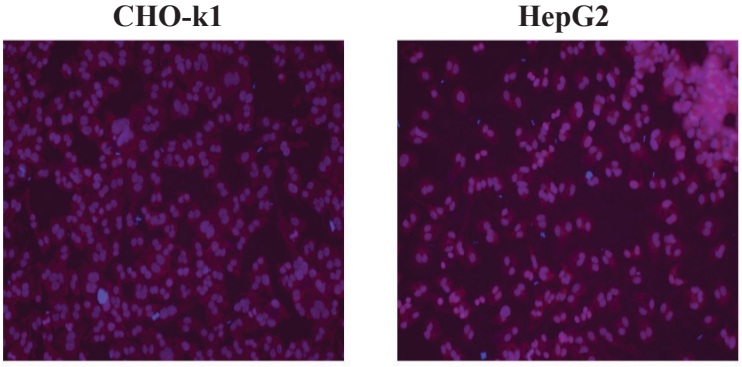

Figure 1. Representative image of the distribution and stretching of CHO-k1 and HepG2 cells in respectively collagen-I and poly-D-lysine coated 96-well plates.

The cell densities used were the most appropriate for each condition to reach at least 1,000 (binuclear) cells per well per plate per 10 image fields. For CHO-k1 cells, compounds were tested in three different conditions, i.e. for 3h with and without S9 mixture and for 24h without S9 mixture. Depending on the condition, 10,000 cells/well were seeded for the 3h condition with S9 mixture (CHO-k1 3h+S9); 5,000 cells /well for the 3h condition without S9 mixture (CHO-k1 3h-S9); and 3,000 cells/well for the 24h condition without S9 mixture (CHO-k1 24h-S9).

HepG2 cells were treated for 24h with the compounds without the addition of S9 mixture as these cells have intrinsic metabolic activity. A protocol for S9 treatment of HepG2 cells was developed and tested for a few compounds, which showed activity in the CHO-k1 cells in the presence of S9 mixture but didn't show activity in the HepG2 cell line. In these cases HepG2 cells were treated for 3h with the compounds in the presence of S9 mixture. HepG2 cells were seeded in a density of 20,000 cells/well for the condition with S9 mixture (HepG2 3h+S9) and in a density of 10,000 cells/well for the condition without S9 mixture (HepG2 24h-S9).

Both CHO-k1 and HepG2 cells were seeded in a total volume of 190 µl for each well and thereafter left in the laminar flow hood for 30 min at RT to obtain an even distribution of the cells over the well surface. The cells were cultured for 24h.

Compound treatment

The cells were first treated with 5 model compounds to determine the effectiveness of the newly developed assays. The reproducubility was tested with taxol and thereafter the assays were evaluated with the 62 compounds from the ECVAM compound list and an additional set of 16 genotoxic references including estrogenic compounds.

Seven serial dilutions (√10 steps) of the compounds or a control sample were added as 10 µl fractions to the cells leading to a final DMSO concentration of 1%. The highest

test concentration of each compound was 1 mM. The different methods that were used for the conditions without and with S9 mixture are described below. Experiments were performed in duplicate on two different 96-well plates. All experiments were repeated at least twice independently.

Conditions without S9 mixture:

CHO-k1 cells were exposed to the compounds for 3h and 24h without the addition of S9 mixture. After these periods, three µg/ml cytochalasin B was added to the cells to block cytokinesis. Cells were cultured for another 24h; thereafter cells were fixed and stained.

HepG2 cells were exposed to the compounds for 24h without the addition of S9. Thereafter, six µg/ml cytochalasin B was added to the cells. HepG2 cells were cultured for 48h; then cells were fixed and stained.

Conditions with S9 mixture:

For the conditions with S9 mixture (CHO-k1 3h+S9 and HepG2 3h+S9), S9 mixture was present during compound exposure (1:10, v/v). S9 mixture consisted of 2.5% S9 liver homogenate from Aroclor-induced Wistar rats (NOTOX, 's Hertogenbosch, The Netherlands), 15.2 mM KCl, 4.28 mM MgCl2, 1.86 mM β-$NADP^+$, 9.29 mM glucose-6-phosphate and 93 mM phosphate buffer pH 7.4. CHO-K1 and HepG2 cells were incubated with S9 mixture for 3h, thereafter medium was removed, and cells were briefly washed with Dulbeco's phosphate buffered saline (PBS) from Gibco (Invitrogen, Carlsbad, CA, USA).

In case of CHO-K1 cells, PBS was replaced by medium containing 5% defined supplemented bovine calf serum and 3 µg/mL cytochalasin B (Sigma-Aldrich, St. Louis, Missouri, USA). After 21h, CHO-k1 cells were fixed and stained.

In case of HepG2 cells, PBS was replaced by culture medium containing 5% defined supplemented bovine calf serum and HepG2 cells were cultured for 21h to allow the cells to divide and recover. Then cytochalasin B was added leading to a final concentration of 6 µg/ml. HepG2 cells were cultured for another 48h and thereafter fixed and stained.

Staining of the micronucleus assay

After compound and cytochalasin B treatment, medium was removed from the cells and the cells were fixed for 20 min at RT by addition of 100 µl 4% paraformaldehyde in PBS. After fixation and washing with PBS, the cells were incubated with 100 µl staining solution for 30 min at RT. The staining solution contained 2 µg/ml Hoechst 33342 (Sigma-Aldrich, St. Louis, Missouri, US) to stain the nuclei and micronuclei and 2 µM DRAQ5 (Biostatus Limited, Shepshed, UK) to stain the cytoplasm. After removal of the staining solution, 100 µl PBS was added to each well.

Imaging and image analysis of the micronucleus assay

Imaging was performed by using an Operetta imaging system equipped with Harmony software version 2.0.1 (Perkin Elmer, Hamburg, Germany). A 20x wide field objective was used to acquire the images. For all conditions without S9 mixture, 10 fields per well were imaged to reach the minimum number of 2,000 binuclear cells needed for analysis. For most treatments without S9 mixture higher numbers were reached. Because cell growth was decreased in the case of conditions with S9 mixture, 20 fields per well were imaged for these conditions.

Analysis of the images was performed using Acapella Studio Suite (Perkin Elmer, Hamburg, Germany). Software for micronucleus analysis was developed in cooperation with Perkin Elmer. Image analysis was based on previously published guidelines for micronuclei scoring [15-17], i.e. both nuclei of similar size and intensity, micronuclei intensity similar to intensity of nucleus, and micronuclei size of 0.0625 up to ≤ 0.33-times the size of the main nuclei. The analysis software contains several modules. A schematic overview of the main modules in the analysis software is given in Figure 2. Firstly, binuclear cells are determined by nuclei detection (column 1) and assessment of nuclear pairing (column 2). Secondly, the cytoplasm of the cells is assessed (column 3). Finally, micronuclei are detected (column 4). Several additional modules were also present to fine-tune the assay, i.e. a module to detect fluorescent compound precipitation (number of fragments outside cell borders), a module to exclude areas with cell clumps ('cloud detection'), and a module to detect apoptotic bodies.

Scoring and calculations of the micronucleus assay

To determine whether a compound was genotoxic or not, the cytotoxicity was taken into account. Two methods were used to assess cytotoxicity. First, the percentage cytotoxicity was calculated based on a decrease of the number of cells after compound treatment:
$((\text{Number of cells})_C - (\text{Number of cells})_T) / (\text{Number of cells})_C * 100\%$
$(\text{Number of cells})_C$ = number of cells in control wells (1% DMSO treated)
$(\text{Number of cells})_T$ = number of cells in compound treated wells.

Second, cytotoxicity was calculated by using the cytotoxicity block proliferation index (CBPI). This method is based on the principle that cytotoxicity often results in cell cycle arrest which is reflected in a decreased ratio of the percentage of binuclear cells to mononuclear cells when using cytochalasin B. The assessment of cytotoxicity by using the CBPI is defined as follows:
$100-100((CBPI_T-1)/ (CBPI_C-1))$
CBPI = (number of mononucleated cells + 2* number of binucleated cells + 3* number of polynucleated cells) / total number of cells
$CBPI_T$ = CBPI of treated cells
$CBPI_C$ = CBPI of control cells (1% DMSO)

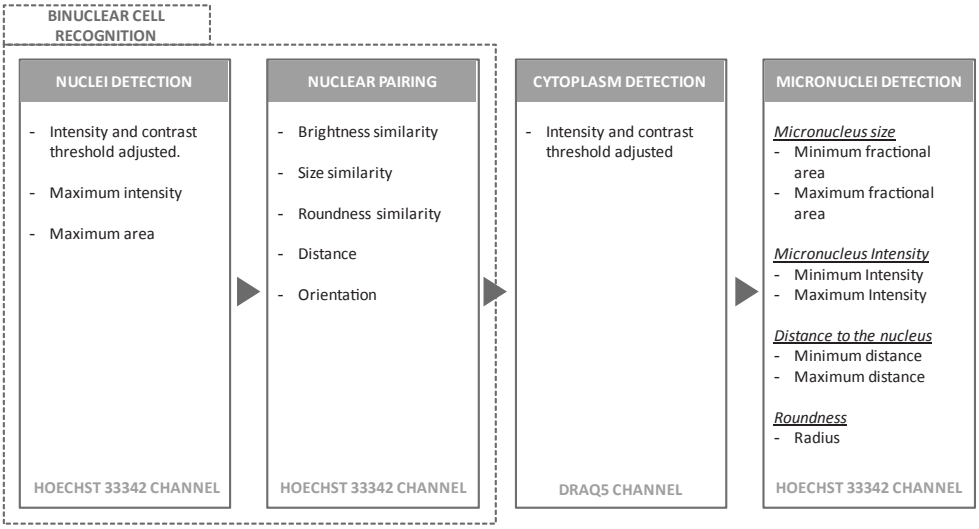

Figure 2. Schematic overview of the HCS in vitro micronucleus assay analysis software. The main modules in the analysis software are used for nuclei detection (column 1), pairing of nuclei (column 2), cytoplasm detection (column 3), and micronuclei detection (column 4). A selection of critical parameters is shown in the four modules.

The following procedure was used for scoring:

1. The sum of the number of micronuclei in binuclear cells in the two duplicate plates was calculated. The same procedure was done for the number of binucleated cells, the number of mononucleated cells, the number of micronuclei in mononucleated cells, and the total number of cells.
2. As main output value, the percentage binuclear cells with micronuclei were calculated: (Number of binuclear cells with micronuclei / number of binuclear cells)*100%.
3. A concentration was defined as positive when the percentage of binuclear cells with micronuclei showed at least a 1.8 fold induction as compared to the percentage in the DMSO (1%) treated cells. This factor was chosen, based on significant fold inductions for all conditions and addition of two times the standard error.
4. Treatments causing compound precipitation were excluded.
5. Treatments causing cytotoxicity in either of the two methods at a level equal to or higher than 80% were excluded.
6. The duplicate values of a positive concentration were checked. When the coefficient of variation was <30% the concentration was defined as positive, if not the result of the treatment in that experiment was considered as equivocal.
7. The second independently performed experiment was scored in the same way as described in points 1-6.
9. When the first experiment showed an equivocal result and the second a positive result,

the compound was considered to be positive.
10. When the first experiment showed an equivocal result and the second a negative result, the compound was considered to be negative.
11. In the case of two experiments that gave an equivocal result, a third test was performed to finally determine whether a compound was positive, negative or equivocal.

After scoring the sensitivity, specificity, predictivity, positive predictive value, and negative predictive value were calculated:
Sensitivity: Percentage of genotoxic compounds (as described in the literature) that tested positive in the high throughput in vitro micronucleus assays.
Specificity: Percentage of non-genotoxic compounds (as described in the literature) that tested negative in the high throughput in vitro micronucleus assays.
Predictivity: Percentage of all tested compounds that was predicted correctly
Positive predictive value: Percentage of compounds that tested positive in the high throughput in vitro micronucleus assays and that are truly genotoxic (as described in the literature).
Negative predictive value: Percentage of compounds that tested negative in the high throughput in vitro micronucleus assays and that are truly non-genotoxic (as described in the literature).

Discrimination of aneugens from clastogens based on size-classification

The area of micronuclei that was calculated in the analysis software, was used to discriminate between small size micronuclei that contain most likely chromosomal fragments (type I micronuclei) and large micronuclei that contain most likely complete chromosomes (type II micronuclei).

The threshold of the fractional area of micronuclei (parameter representing micronuclei size) for discrimination between type I and II micronuclei was assessed for CHO-k1 cells. MMS and taxol were used to set this threshold, as MMS and taxol have a strong clastogenic and aneugenic mode of action, respectively. The results are presented in Figure 3. As can be seen the most optimal threshold that gives the best discrimination between taxol and MMS seems to be 0.17 in CHO-k1 cells (the same threshold was used for HepG2 cells). The percentage type II micronuclei belonging to this threshold that separates both compounds is 30% (Figure 3). Based on these results it was defined that compounds with a median above 30% are classified as aneugens and compounds with a median less or equal to 30% are classified as clastogens.

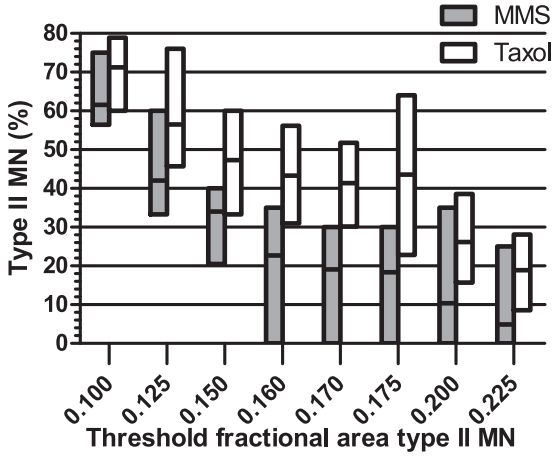

Figure 3. Discrimination of clastogens (i.e. MMS) from aneugens (i.e. taxol) by using different fractional area thresholds. The percentage type II micronuclei at different thresholds are shown. The borders of the bars represent the maximum and minimum values and the line within the bar represents the median.

Results

Development of a HCS in vitro micronucleus assay

The automated analysis software was developed and optimized in collaboration with Perkin Elmer. An example of image analysis in the case of CHO-k1 cells is shown in Figure 4, which shows the main steps in the image analysis. Firstly, micronuclei are detected and paired, secondly the cytoplasm of the cells is detected, and finally the micronuclei are detected.

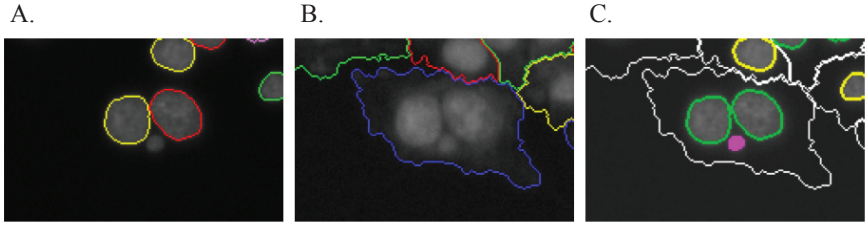

Figure 4. Representative images of the micronucleus image analysis software for CHO-k1 cells. The three main steps during image analysis are shown, i.e. the detection and pairing of nuclei (A), the detection of the cytoplasm (B), and detection of micronuclei within the cytoplasm of a binuclear cell depicted as a pink dot (C).

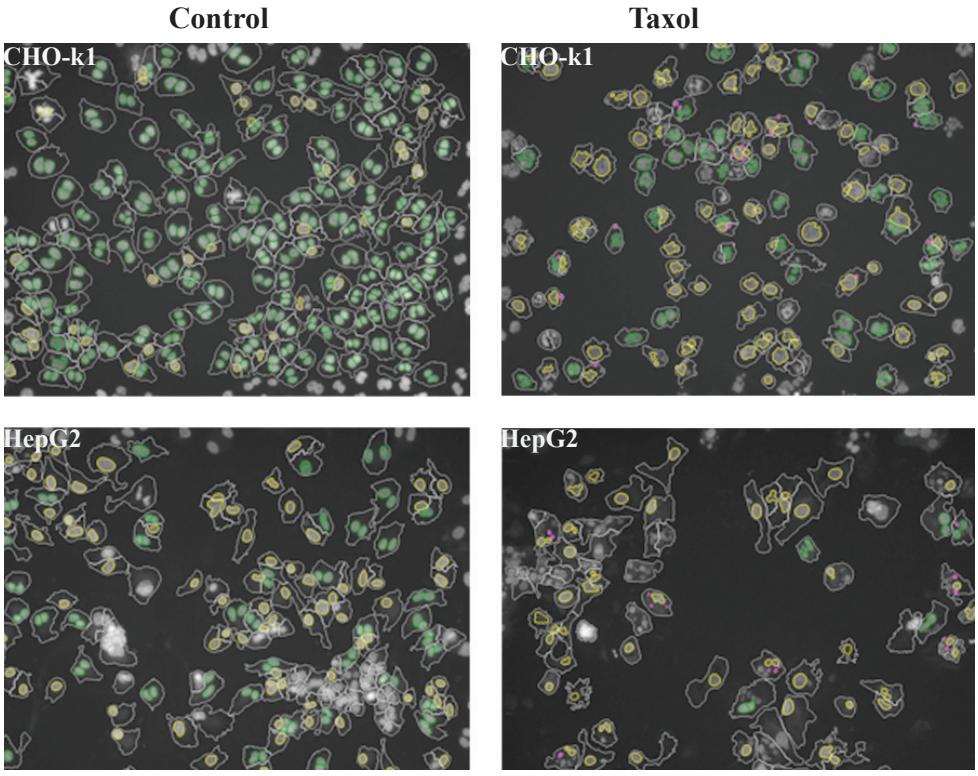

Figure 5. Representative results of the image analysis of control and taxol treated CHO-k1 and HepG2 cells. Mononucleated (yellow nuclei) and binucleated cells (green nuclei) can be distinguished. Additionally, no micronuclei are observed within the control image field whereas in the image fields of the treated cells many micronuclei (pink dots) can be observed.

Figure 5 shows an example of the final result of image analysis of control cells (1% DMSO treated) and $1.00x10^{-5}$ M taxol treated cells for both CHO-k1 and HepG2 cells. During analysis a differentiation is made between mononuclear and binuclear cells and micronuclei are indicated as pink dots. Multiple pink dots can be seen after treatment with the genotoxic compound taxol.

Testing of model compounds and reproducibility

To get a first impression about the effectiveness of the assays, five model compounds were tested (i.e. aflatoxin B1, benzo[a]pyrene (B[a]P), cyclophosphamide, 7,12-dimethylbenzanthracene (DMBA), and MMS). The concentration dependent fold induction of the fraction of binuclear cells with micronuclei after exposure to these compounds is shown in Figure 6 for the CHO-k1 (3 conditions) and HepG2 HCS IVMN assays.

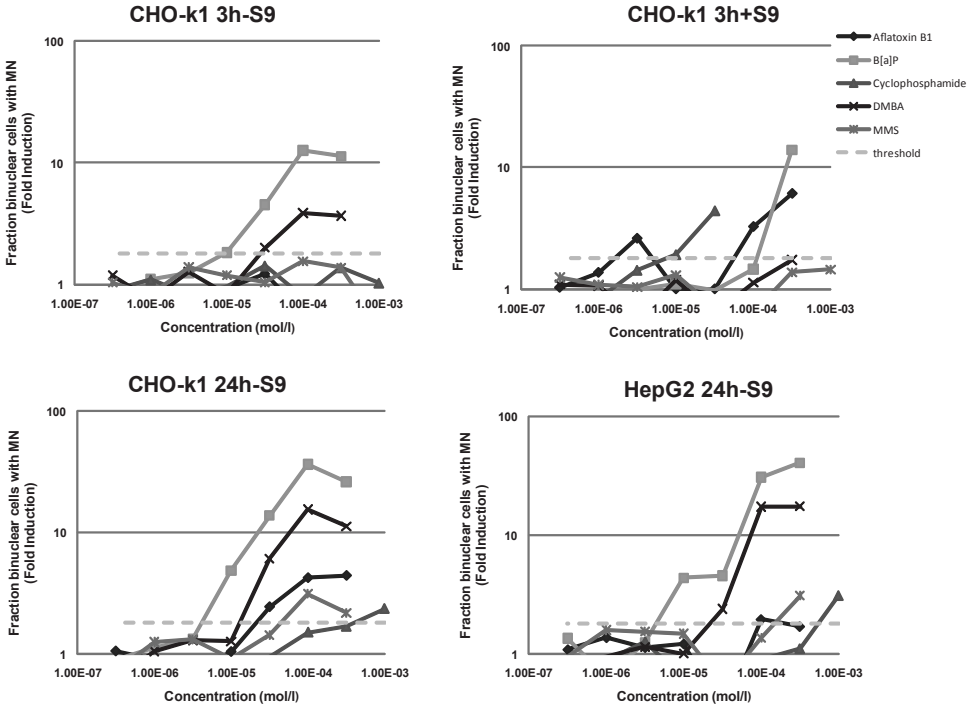

Figure 6. HCS in vitro micronucleus assay in CHO-k1 and HepG2 cells. The dose response curves of five model compounds are shown, i.e. aflatoxin B1, benzo[a]pyrene (B[a]P), cyclophosphamide, 7,12-dimethylbenzanthracene (DMBA), and MMS. The dashed line indicates the genotoxicity threshold (see M&M).

In the CHO-k1 3h-S9 condition, only B[a]P and DMBA showed an effect above the genotoxicity threshold. The lowest effective concentration (LEC) was in both cases $3.16x10^{-5}$ M. As both compounds are proximate genotoxins that are activated by enzymes of the cytochrome P450 (CYP) 1A or CYP1B family, these enzymes appear to be active in the CHO-k1 cell line used.

The addition of S9 mixture (CHO-k1 3h+S9) metabolically activated the proximate genotoxins aflatoxin B1 and cyclophosphamide, which are respectively activated by CYP3A4 and CYP2B6. The LEC of aflatoxin B1 was $1.00x10^{-4}$ M and that of cyclophosphamide $1.00x10^{-5}$ M. Both B[a]P and DMBA showed a much lower activity after the addition of S9 mixture in comparison to the 3h condition without S9 mixture, the effect of DMBA was even just below the threshold. B[a]P had a LEC of $3.16x10^{-4}$ M. MMS did not show any effect.

In the CHO-k1 24h-S9 condition all compounds showed a genotoxic effect. B[a]P was the most potent compound with an LEC of $1.00x10^{-5}$ M. Aflatoxin B1 and DMBA had an LEC of $3.16x10^{-5}$ M. MMS and cyclophosphamide were the weakest active compounds

with a LEC of $1.00x10^{-4}$ and $1.00x10^{-3}$M, respectively. As with exception of MMS, the compounds are proximate genotoxins, enzyme systems that activate these compounds must be present in the cells.

All five compounds showed also a genotoxic effect in the HepG2 cell line. The potency of the compounds showed almost the same order as in the CHO-k1 cell line in the CHO-k1 24h-S9 condition.

The reproducibility of the genotoxicity determination in the HCS in vitro micronucleus assays was investigated using taxol as reference compound (Figure 7). Four independent experiments were performed except for the CHO-K1 3h-S9 condition, for which only three replicates were performed. For the CHO-k1 3h-S9 condition the LEC was one time $3.16x10^{-7}$ M and two times $1.00x10^{-6}$ M. For the CHO-k1 3h+S9 condition the LEC was reproducible with two times $3.16x10^{-5}$ M and two times $1.00x10^{-4}$ M. For the 24h incubation condition, the LEC value was two times $1.00x10^{-7}$ M and two times $1.00x10^{-6}$ M

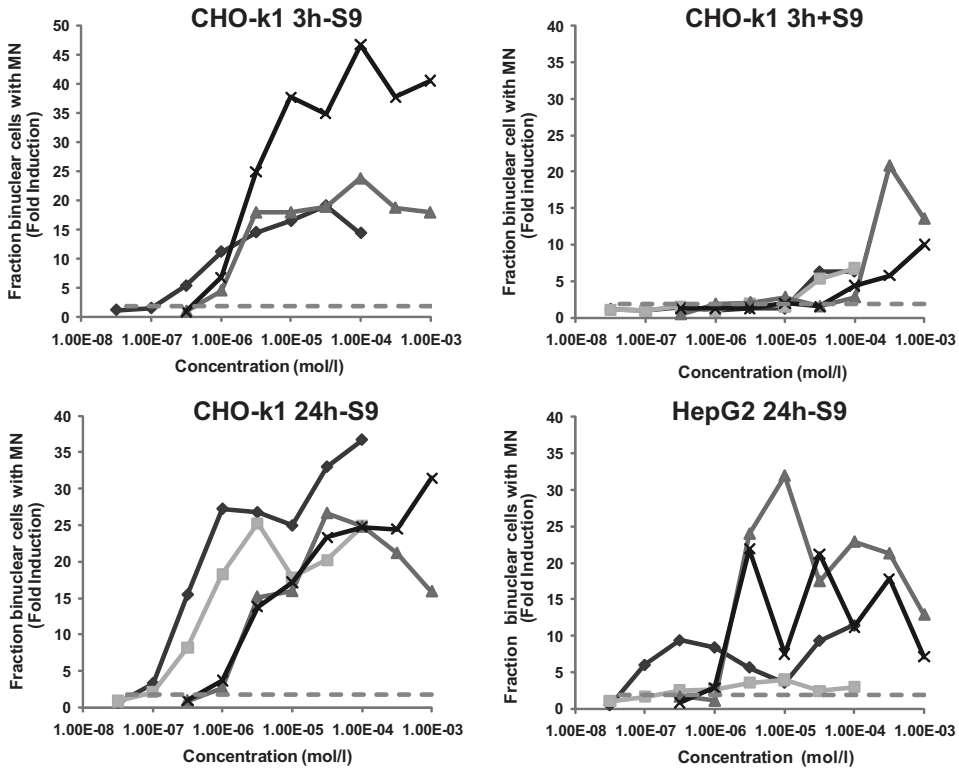

Figure 7. Reproducibility of the genotoxic effect of taxol in the HCS in vitro micronucleus assay for CHO-k1 and HepG2 cells. Three or four independent experiments were performed. The dashed line indicates the genotoxicity threshold (see M&M). Variation in the LEC is limited. At higher concentrations there is more variation due to high levels of cytotoxicity (see Figure 8).

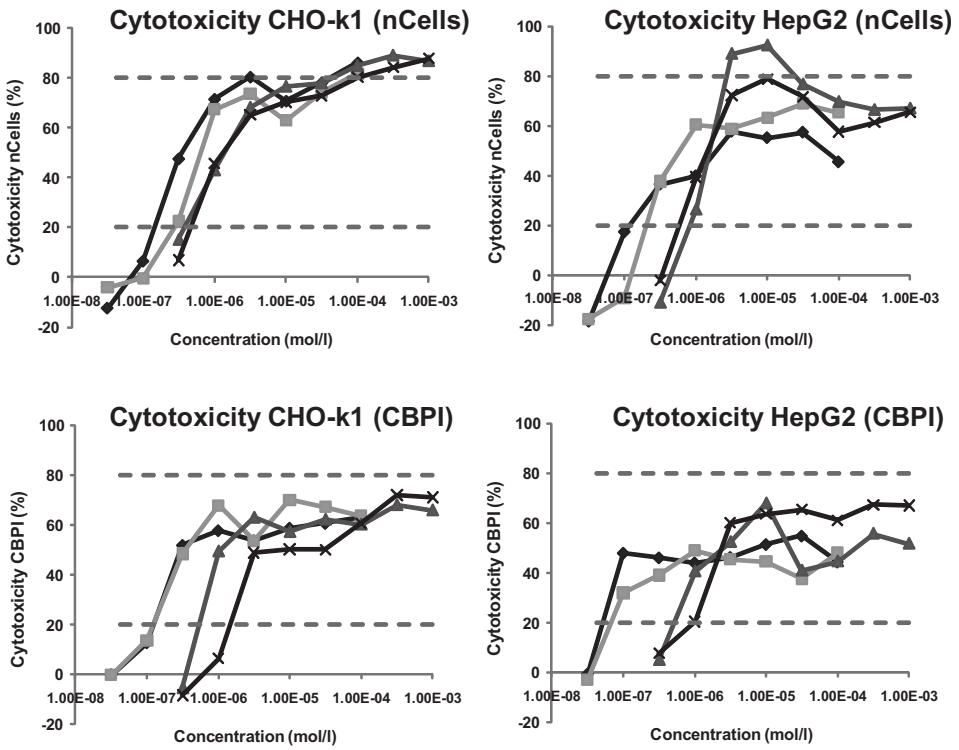

Figure 8. Reproducibility of the cytotoxic effect of taxol based on number of cells (nCells) and CBPI in the HCS in vitro micronucleus assay for CHO-k1 (CHO-k1 24h-S9 condition) and HepG2 cells. The two dashed line at 20% and 80% cytotoxicity represent the minimal toxic concentration and toxicity level above which genotoxicity measurement are excluded (see M&M), respectively.

For the CHO-k1 3h-S9 condition the LEC was one time $3.16x10^{-7}$ M and two times $1.00x10^{-6}$ M. For the CHO-k1 3h+S9 condition the LEC was reproducible with two times $3.16x10^{-5}$ M and two times $1.00x10^{-4}$ M. For the 24h incubation condition, the LEC value was two times $1.00x10^{-7}$ M and two times $1.00x10^{-6}$ M.

The variation in the HepG2 HCS in vitro micronucleus assay was larger. The LEC of taxol was different in all four experiments, i.e. $1.00x10^{-7}$ M, $3.16x10^{-7}$ M, $1.00x10^{-6}$ M and $3.16x10^{-6}$ M.

The variation in the maximum induction between the experiments was much larger. This was due to cytotoxicity. For concentrations higher than $1.00x10^{-6}$ M the cytotoxicity of taxol rapidly increased to 60-80% for the conditions without S9 (see also Figure 8). Cytotoxicity was less in the presence of S9 mixture.

The reproducibility of the two methods to assess cytotoxicity was also tested (Figure 8). This was tested for taxol in both the CHO-k1 (CHO-k1 24h-S9 condition) and HepG2 cell line (HepG2 24h-S9).

Cytotoxicity measurement based on the number of cells (nCells) showed the lowest variation. In CHO-k1 cells, the minimum toxic dose (MTD; 20% cytotoxicity) was two times $1.00x10^{-6}$ M and two times $3.16x10^{-7}$ M. The MTD in HepG2 cells was similar with three times $1.00x10^{-6}$ M and one time $3.16x10^{-7}$ M.

Cytotoxicity based on the CBPI showed more variation. The MTD was in CHO-k1 cells one time $3.16x10^{-6}$ M, one time $1.00x10^{-6}$ M, and two times $3.16x10^{-7}$ M. In HepG2 cells the MTD was two times $1.00x10^{-6}$ M and two times $1.00x10^{-7}$ M.

Overall the experiments in this section showed that reference compounds gave the expected genotoxic effects. In addition, the reproducibility of the HCS in vitro micronucleus assays is good. Therefore the assays were further validated by using a larger set of genotoxic and non-genotoxic compounds.

Validation with the ECVAM compound list

The HCS in vitro micronucleus assays in CHO-k1 and HepG2 cells were validated by testing the 62 ECVAM compounds. The overall results are shown in Tables 1-3.

Of the 20 genotoxic compounds (Table 1) ENU, dimethylnitrosamine, 2,4-diaminotoluene, and hydroquinone were tested negative in the CHO-K1 cell line. Eight genotoxins tested negative in the HepG2 cell line. Like in the CHO-k1 cells, ENU and hydroquinone were tested negative. Another six compounds were also tested negative, i.e. 2-acetylaminofluorene, IQ, cadmium chloride, p-chloroaniline, azidothymidine, and chloramphenicol.

Of the 19 non-genotoxic compounds that have been reported to show often false positive results in mammalian in vitro genotoxicity assays (Table 2), three showed a positive result in CHO-k1 cells, i.e. ethionamide, propyl gallate and 2,4-dichlorophenol. None of these 19 compounds gave a positive result in HepG2 cells.

Of the 23 non-DNA-reactive chemicals that have not been reported to give positive results in vitro (Table 3), two compounds were positive in the CHO-k1 assay, i.e. N-dicyclohexyl thiourea and progesterone. In HepG2 cells five of these 23 compounds showed a positive result, i.e. phenformin HCl, ephedrine sulfate, phenanthrene, progesterone (+S9) and tris(2-ethylhexyl)phosphate.

Table 1. HCS IVMN assays regarding the ECVAM compounds that were defined as in vivo genotoxins. False negative results are indicated in bold.

Chemical	CAS number	Further information	CHO-k1	LEC (mol/l)	Induction	HepG2	LEC (mol/l)	Induction
I. Ames-positive in vivo genotoxins								
(i) O^6 and N^7 alkylators								
Cyclophosphamide	6055-19-2	Requires metabolic activation (CYP2B6)	Y (S9)[a]	$1.00x10^{-5}$	4.39	Y	$3.16x10^{-5}$	3.09
ENU	759-73-9	Strong gene mutagen (O^6 alkylation)	**N**			**N**		
MMS	66-27-3	Strong clastogen (N^7 alkylation)	Y	$1.00x10^{-4}$	3.48	Y	$1.00x10^{-4}$	3.26
(ii) Polycyclic aromatic hydrocarbons								
Benzo[a]pyrene	50-32-8	Requires metabolic activation (CYP 1A1; 1B1, epoxide hydrolase); forms bulky adducts	Y	$1.00x10^{-5}$	36.15	Y	$1.00x10^{-5}$	30.71
7,12-Dimethylbenzanthracene	57-97-6	Requires metabolic activation (CYP1B1); forms bulky adducts	Y	$3.16x10^{-5}$	15.37	Y	$3.16x10^{-5}$	17.30
(iii) Aromatic amines								
Dimethylnitrosamine	62-75-9	Alkylating agent after activation by CYP2E1(which is not highly expressed in rat liver S9): produces O^6- and N^7-methyl guanine adducts	**N**			Y	$1.00x10^{-4}$	2.70
2-Acetylaminofluorene	53-96-3	Hydroxylated by CYP1A2 and then acetylated. Forms C8 adduct on guanine	Y (S9)[a]	$1.00x10^{-4}$	5.60	**N**		
2,4-Diaminotoluene	95-80-7	Aromatic amine, requires metabolic activation	**N**			Y	$3.16x10^{-4}$	4.38
IQ (2-amino-3methyl imidazo[4,5-*f*]quinoline)	76180-96-6	Heterocyclic amine with potent genotoxicity, requires metabolic activation	Y (S9)[a]	$3.16x10^{-5}$	2.55	**N**		
PhIP.HCl (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine		Heterocyclic amine with potent genotoxicity, requires metabolic activation	Y (S9)[a]	$1.00x10^{-4}$	2.97	Y	$3.16x10^{-4}$	1.93
(iv) Others								
Aflatoxin B1	1162-65-8	Activated by CYP3A4, which is not highly expressed in rats compared with humans. Forms various adducts	Y (S9)[a]	$3.16x10^{-6}$	18.89	Y	$1.00x10^{-5}$	1.96
Cadmium chloride	10108-64-2	Inorganic carcinogen	Y	$1.00x10^{-6}$	4.05	**N**		
Cisplatin	15663-27-1	Cross-linking agent	Y	$1.00x10^{-4}$	5.30	Y	$3.16x10^{-4}$	6.03
p-Chloroaniline	106-47-8	No adducts	Y (S9)[a]	$1.00x10^{-4}$	4.30	**N**		
II. In vivo genotoxins negative or equivocal in Ames								
Etoposide	33419-42-0	Topoisomerase inhibitor	Y	$1.00x10^{-7}$	10.31	Y[b]	$3.16x10^{-6}$	2.66
Hydroquinone	123-31-9	MOA: aneugen	**N**			**N**		
Azidothymidine	30516-87-1	MOA: nucleoside analogue	Y (S9)[a]	$1.00x10^{-4}$	3.66	**N**		
Sodium arsenite	7784-46-5	Inorganic carcinogen MOA: oxidant? repair inhibitor?	Y	$3.16x10^{-5}$	3.34	Y	$1.00x10^{-6}$	7.89
Taxol	33069-62-4	MOA: aneugen	Y	$1.00x10^{-6}$	46.73	Y	$3.16x10^{-6}$	22.88
Chloramphenicol	56-75-7	MOA: clastogen that binds to DNA	Y (S9)[a]	$1.00x10^{-5}$	6.33	**N**		

[a] Compounds that are activated by S9 mixture but already show some activity without the addition of S9 mixture

[b] Detoxification due to addition of S9 mixture to HepG2 cells activated the compound.

Table 2. HCS IVMN assays regarding the ECVAM compounds that were defined as non-DNA reactive chemicals but have been reported to induce chromosome aberrations or tk mutations in mouse lymphoma cells, often at high concentrations or at high levels of cytotoxicity. False positive results are indicated in bold.

Chemical	CAS number	CHO-k1	LEC (mol/l)	Induction	HepG2	LEC (mol/l)	Induction
I. Non-carcinogens that are negative or equivocal for genotoxicity in vivo							
D,L-Menthol	15356-70-4	N			N		
Phthalic anhydride	85-44-9	N			N		
Tertiary-butylhydroquinone	1948-33-0	N			N		
o-Anthranilic acid	118-92-3	N			N		
1,3-Dihydroxybenzene (resorcinol)	108-46-3	N			N		
2-Ethyl-1,3-hexanediol	94-96-2	N			N		
Sulfisoxazole	127-69-5				N		
II. Non-carcinogens with no in vivo genotoxicity data							
Ethionamide	536-33-4	**Y**	$1.00x10^{-4}$	3.19	N		
Curcumin	458-37-7	N			N		
Benzyl alcohol	100-51-6	N			N		
Urea	57-13-6	N			N		
III. Non-genotoxic carcinogens or carcinogenic by irrelevant (for humans) mechanism							
Sodium saccharin	128-44-9	N			N		
IV. Supplementary list (prediction of in vitro genotoxicity results less clear)							
Propyl gallate	121-79-9	**Y (S9)**[a]	$1.00x10^{-4}$	3.30	N		
p-Nitrophenol	100-02-7	N			N		
Sodium xylene sulfonate	1300-72-7	N			N		
Ethyl acrylate	140-88-5	N			N		
Eugenol	97-53-0	N			N		
Isobutyraldehyde	78-84-2	N			N		
2,4-Dichlorophenol	120-83-2	**Y (S9)**			N		

[a] Compounds that are activated by S9 mixture but already show some activity without the addition of S9 mixture

Table 3. HCS IVMN assays regarding the ECVAM compounds that were defined as non-DNA-reactive chemicals (including non-genotoxic carcinogens). False positive results are indicated in bold.

Chemical	CAS number	CHO-k1	LEC (mol/l)	Induction	HepG2	LEC (mol/l)	Induction
I. Non-carcinogens with negative in vivo genotoxicity data							
Ampicillin trihydrate	7177-48-2	N			N		
D-mannitol	69-65-8	N			N		
II. Non-carcinogens with no in vivo genotoxicity data							
Phenformin HCl	834-28-6	N			**Y**	$3.16x10^{-4}$	3.16
n-Butyl chloride	109-69-3	N			N		
(2-chloroethyl)trimethyl-ammonium chloride	999-81-5	N			N		
Cyclohexanone	108-94-1	N			N		
N,N-dicyclohexyl thiourea	1212-29-9	**Y**	$3.16x10^{-5}$	2.13	N		
Trisodium EDTA trihydrate	150-38-9	N			N		
Ephidrine sulphate	134-72-5	N			**Y**	$1.00x10^{-3}$	4.54
Erythromycin stearate	643-22-1	N			N		
Fluometron	2164-17-2	N			N		
Phenanthrene	85-01-8	**N**			**Y**	$1.00x10^{-4}$	4.76
III. Non-genotoxic carcinogens							
D-Limonene	5989-27-5	N			N		
Di-(2-ethylhexyl)phthalate	117-81-7	N			N		
Amitrole	61-82-5	N			N		
Tert-butyl alcohol	75-65-0	N			N		
Diethanolamine	111-42-2	N			N		
Melamine	108-78-1	N			N		
Methyl carbamate	598-55-0	N			N		
Progesterone	57-83-0	**Y (S9)**[a]	$3.16x10^{-6}$	3.04	**Y**[b]	$1.00x10^{-3}$	1.92
Pyridine	110-86-1	N			N		
Tris(2-ethylhexyl)phosphate	78-42-2	N			**Y**	$1.00x10^{-3}$	2.76
Hexachloroethane	67-72-1	N			N		

[a] Compounds that are activated by S9 mixture but already show some activity without the addition of S9 mixture

[b] Detoxification due to addition of S9 mixture to HepG2 cells activated the compound.

Table 4. Validation of the CHO-k1 and HepG2 HCS IVMN assays with respect to the ECVAM compound list.

	CHO-k1 (%)	HepG2 (%)
Sensitivity	80 (16/20)	60 (12/20)
Specificity	88 (37/42)	88 (37/42)
Predictivity	85 (53/62)	79 (49/62)
Positive predictive value	76 (16/21)	75 (12/17)
Negative predictive value	90 (37/41)	82 (37/45)

The sensitivity, specificity, predictivity, positive predictive value and negative predictive value were calculated as shown in Table 4. The sensitivity of the HCS IVMN assay in CHO-k1 cells was with 80% (16/20) higher than the sensitivity of 60% (12/20) that was observed for HepG2 cells. The number of false positive results in both tests was low with a specificity of 88% (37/42). The scores resulted in an overall predictivity of the assay in CHO-k1 and HepG2 cells of 85% (53/62) and 79% (49/62), respectively.

In addition, the positive and negative predictive values were calculated for the HCS IVMN assay in both cell lines. The positive predictive values were 76% (16/21) and 71% (12/17) for the CHO-K1 and HepG2 assay, respectively. Negative predictive values were 90% (37/41) for the CHO-k1 and 82% (37/45) for the HepG2 assay.

Testing of 16 additional genotoxic compounds

An additional set of 16 genotoxic compounds, including several estrogenic compounds, were tested in both the CHO-k1 and HepG2 HCS IVMN assays (Table 5). Of the 16 genotoxic compounds, 11 gave a positive result in the CHO-k1 HCS IVMN assay (69%). Like observed with the compounds from the ECVAM list, the sensitivity of the HepG2 HCS IVMN assay was lower as here only nine compounds showed to be positive (56%). For most genotoxic compounds the fold induction was lower in the HepG2 cell line. Of the compounds that were genotoxic in the CHO-k1 HCS IVMN assay, three compounds were negative in the HepG2 cell line, i.e. 2,7-dinitrofluorene, 4-hydroxy-estrone, and cytarabine. Methotrexate was genotoxic in HepG2 cells but showed no genotoxicity in the CHO-k1 cell line.

Discrimination of aneugens from clastogens based on size-classification

Two types of micronuclei were defined, i.e. type I micronuclei containing chromosomal fragments and type II micronuclei containing complete chromosomes. Based on the size of micronuclei the genotoxic compounds can be classified as clastogens (percentage type II micronuclei<30%) or aneugens (percentage type II micronuclei>30%). See M&M section.

Table 5. Results from the HCS IVMN assays regarding an additional set of 16 genotoxic compounds. False negative results are indicated in bold.

Chemical	CAS number	Further information	Mammalian gentox in vitro	Mammalian gentox in vivo	CHO-k1	LEC (mol/l)	Induction	HepG2	LEC (mol/l)	Induction
I. Genotoxic compounds showing bacterial mutagenicity										
2,7-Dinitrofluorene	5405-53-8		Y	Y	Y (S9)	$1.00x10^{-3}$	1.90	**N**		
2-Hydroxy-estrone	362-06-1	Catechol estrogen	Y	Y	**N**			**N**		
2-Methoxy-estradiol	362-07-2	Catechol estrogen	Y	Y	Y (S9)[a]	$1.00x10^{-5}$	10.28	Y	$1.00x10^{-5}$	10.50
3-Methylcholanthrene	56-49-5		Y	Y	Y (S9)[b]	$1.00x10^{-6}$	15.8	Y	$1.00x10^{-5}$	16.42
4-Hydroxy-estrone	3131-23-5	Catechol estrogen	Y	Y	Y	$3.16x10^{-5}$	2.03	**N**		
Dacarbazine	224-396-1		Y	ND	**N**			**N**		
Doxorubicin	25316-40-9	Topoisomerase inhibitor	Y	Y	Y	$1.00x10^{-7}$	12.56	Y	$1.00x10^{-7}$	5.66
Ellipticin	519-23	Topoisomerase inhibitor	ND	Y	Y (S9)[a]	$1.00x10^{-6}$	7.63	Y	$1.00x10^{-6}$	5.08
Melphalan	148-82-3		Y	ND	Y (S9)[a]	$1.00x10^{-6}$	8.75	Y	$1.00x10^{-6}$	2.80
Salicylamide	65-45-2		Y	Y	**N**			**N**		
Uramustine	66-75-1		ND	ND	Y (S9)[a]	$3.16x10^{-6}$	8.81	Y	$1.00x10^{-6}$	7.19
II. Genotoxic but bacterial mutagenicity negative or not defined										
Cytarabine	147-94-4	Anti-metabolite	Y	Y	Y	$3.16x10^{-4}$	4.36	**N**		
Diethylstilbestrol		Estrogen	Y	Y	**N**			**N**		
Estradiol-17β	50-28-2	Estrogen	Y	Y	Y	$3.16x10^{-4}$	13.42	Y	$3.16x10^{-4}$	7.78
Ethinylestradiol-17β	57-63-6	Estrogen	Y	Y	Y	$1.00x10^{-4}$	9.00	Y	$3.16x10^{-5}$	4.53
Methotrexate	59-05-2	Anti-metabolite	Y	Y	**N**			Y	$3.16x10^{-5}$	1.92

[a] Compounds that are activated by S9 mixture but already show some activity without the addition of S9 mixture

All compounds that showed a genotoxic response in the CHO-k1 HCS IVMN assay were classified as clastogens or aneugens. The results are presented in Figure 9. Most compounds have a percentage type II micronuclei with a median that is lower than 30%, indicating that these compounds have most likely a clastogenic mode of action. The percentage type II among these clastogenic compounds has a wide range of 0% for cisplatin up to 26.8% for IQ which reflects clastogens giving small and larger chromosomal fragments. The variation in the percentage of type II micronuclei for sodium arsenite made classification of this compound difficult. The compounds that were classified as aneugenic are propyl gallate, taxol, 17β-estradiol, 2-methoxy-estradiol, 2,4-dichlorophenol, 17β-ethinylestradiol and chloramphenicol.

As the total number of binuclear cells per 10 fields was lower for HepG2 cells and in general also the induction factors were lower, it was difficult to do the same classification for all genotoxic compounds in the HepG2 cell line. However, to show that the principle works in HepG2 cells, the number of type II micronuclei was assessed for the strong aneugen taxol; and for B[a]P and DMBA which were (strong) clastogens in the CHO-k1 cell line (Figure 10). The results clearly show that taxol gives like in the CHO-k1 cell line a high percentage of type II micronuclei; while B[a]P and DMBA give like in CHO-k1 cells a low percentage of type II micronuclei and are therefore most likely clastogens. These results show that the classification method also can be used for the HepG2 cell line.

Usage of S9 mixture to explain differences between results in the HCS IVMN assays in CHO-k1 and HepG2 cells.

Several compounds that were activated in the CHO-k1 3h+S9 condition, showed no activity in the HepG2 cell line. It was investigated whether the addition of S9 mixture to HepG2 cells would result in a genotoxic effect in this cell line as well. The HCS IVMN assay for the HepG2 condition with S9 mixture was performed for eleven compounds, i.e. cyclophosphamide, ENU, 7,12-diaminotoluene, IQ, cadmium chloride, p-chloroaniline, etoposide, hydroquinone, azidothymide, chloramphenicol and progesterone. Moreover, aflatoxin B1 was used to test the S9 protocol.

The results are shown in Figure 11. The application of S9 mixture resulted in a 10-fold potentiation of the genotoxic effect of aflatoxin B1, indicating that the protocol that was used in case of CHO-k1 cells is also applicable for the HepG2 cell line. In the presence and absence of S9 mixture the cytotoxicity of aflatoxin B1 remained unchanged. Only for two of the eleven compounds an effect on the induction of micronuclei with S9 mixture was observed.

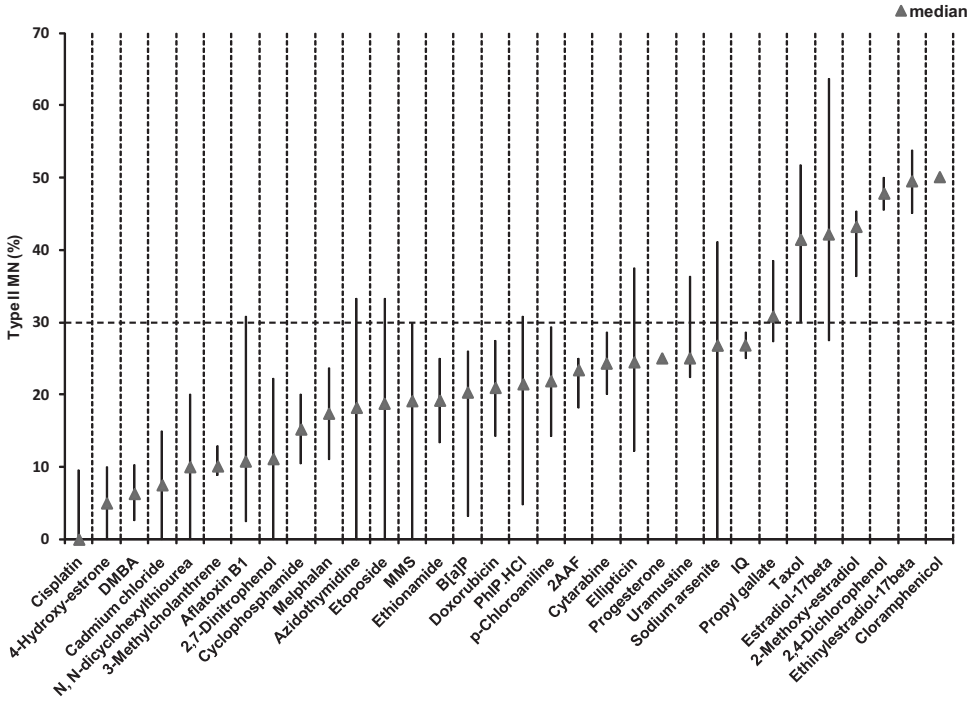

Figure 9. The percentage type II micronuclei (MN) for genotoxins in the CHO-k1 HCS IVMN assay. Concentrations causing an induction of MN were used for the asesssment of type II MN. The top of the lines represents the maximum, the bottom the minimum, and the triangle the median. Compounds with a median higher than 30% were defined as compounds with an aneugenic mode of action (see M&M); compounds with a median less or equal than 30% were defined as clastogens (horizontal dashed line).

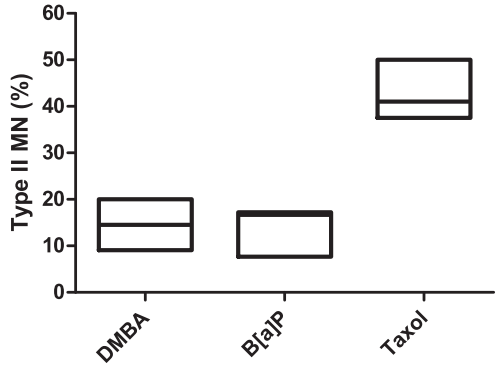

Figure 10. The percentage type II micronuclei for 7,12-Dimethylbenzanthracene (DMBA), benzo[a] pyrene (B[a]P) and taxol determined in the HCS IVMN assay in HepG2 cells. The borders of the bars represent the minimum and maximum values and the line within the bars the median.

Etoposide showed no genotoxic effect in the absence of S9 mixture. In this condition there was already 70% cytotoxicity at a concentration of $1.00x10^{-6}$ M. Addition of S9 mixture resulted in a large decrease of cytotoxicity as now a 100-fold higher concentration of $1.00x10^{-4}$ M etoposide was needed to reach this level of cytotoxicity. A clear genotoxic effect was observed at a concentration of $3.16x10^{-6}$ M.

Progesterone was not genotoxic in the absence of S9 mixture and the compound was cytotoxic at a concentration of $1.00x10^{-5}$ M. Addition of S9 mixture clearly resulted in detoxification and a genotoxic effect was now observed at the highest test concentration of $1.00x10^{-3}$ M.

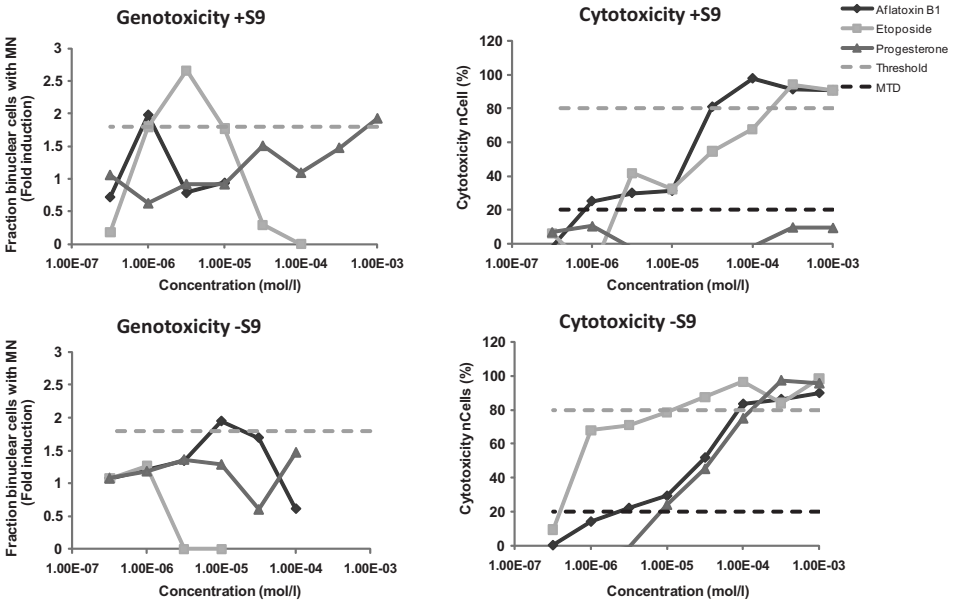

Figure 11. Effect of S9 mixture in the HepG2 HCS IVMN assay. For an explanation of the thresholds see M&M.

Discussion

Development and optimization of HCS IVMN assays

Two HCS IVMN assays have been developed, one in the CHO-k1 and one in the HepG2 cell line. Imaging and scoring of the number of MN in these assays has a much higher throughput than manual scoring of MN in microscopic slides. Imaging of 10 fields per well for one 96-well plate takes 50 min. Analysis of these 960 images takes 3h. Depending on the hardware configuration it is possible to perform up to six

analyses in parallel. Compared to the HCS assay that was described by Diaz et al. [2], the throughput of the present assays is higher. In their assay it took 12h to image and analyze 11 compounds in duplicate, which would with our assay method in the most simple hardware configuration take about 8h.

In addition, the reproducibility of the assays was good and two relatively cheap as well as up to at least eight months stable fluorescent stains were used. The cytoplasmic stain used by Diaz et al. [2] was not that stable as plates had to be measured within three days after staining.

Validation with the ECVAM compound list

The HCS IVMN assays were validated by using 62 chemicals that were described in an ECVAM compound list that can be used for the optimization or validation of in vitro genotoxicity assay [13].

The specificity of both cell lines was high with 88% (37/42). In both assays only five compounds out of the 42 non-genotoxic compounds showed a positive result. This specificity is much higher than the specificity score of 30.8% for carcinogenicity that was reported by Kirkland et al. [3]. In the study described by Kirkland, the specificity was based on a relative small number of 26 non-carcinogenic compounds. There was almost no overlap of compounds with the present study; only for propyl gallate, which was positive in the present study, an equivocal result was reported. The difference in compound set or method of scoring might have been the reason, but the large difference is unexpected. The HCS IVMN assay described by Diaz et al. had also a high specificity of 100%. However, in this study only 13 non-genotoxic compounds were tested which is a relatively small number. None of these 13 compounds were tested in the present study.

The 42 non-genotoxic compounds that were tested contained 19 compounds that are known to give often false positive results in mammalian in vitro genotoxicity assays. Of these 19 compounds, only three gave a positive score in the CHO-k1 cell line, i.e. ethionamide, propyl gallate, and 2,4-dichlorophenol. Both propyl gallate and 2, 4-dinitrophenol were activated by the addition of S9 mixture. None of the 19 compounds showed a positive result in the HepG2 cell line.

Of the remaining 23 non-genotoxic compounds, two gave a false positive result in the CHO-k1 cell line, i.e. N,N-dicyclohexyl thiouria and progesterone which is activated by S9 mixture. For progesterone it has been shown that it is a weak inducer of MN in the liver [18]. In HepG2 cells five of these 23 compounds showed a positive result. Three were in the subgroup of non-carcinogens with no in vivo genotoxicity data, i.e. phenformin HCl, ephidrine sulphate, and phenanthrene. The other two positive results were in the group of non-genotoxic carcinogens; like in CHO-k1 cells progesterone showed a weak positive result (+S9 mixture) and in addition tris(2-ethylhexyl)phosphate was positive.

The low number of false positive results is also reflected by the high negative predictive values of 90% and 82% for the CHO-k1 and HepG2 HCS IVMN assays. These low numbers of false positive results make both assays suitable candidates for implementation in the early phases of drug development, since not too many compounds will be excluded from further development based on false positive genotoxicity results.

Also the sensitivity of the assays needs to be high for implementation of the assays. The sensitivity of the assay in CHO-k1 cells was high with 80% (16/20), which is comparable to the sensitivity of 78.7% reported by Kirkland et al. [3] and that of the automated in vitro micronucleus assay in CHO-K1 cells as developed by Diaz et al., which obtained 88% sensitivity [2]. Further investigation of the four compounds that gave a false negative result (i.e. ENU, dimetylnitrosamine, 2,4-diaminotoluene, hydroquinone) shows that dimethylnitrosamine is metabolized by CYP2E1 [13]. This CYP is not highly expressed in rat liver S9 which might explain the negative result. For the three other compounds no clear explanation for the negative result can be given.

The sensitivity of the HCS assay in HepG2 cells was lower with 60% (12/20). The relatively low levels of phase I enzymes in combination with normal levels of phase II enzymes might be the reason for the lower sensitivity, as metabolism might shift to detoxification [11, 12]. Moreover, the HepG2 cell line contains a functionally active p53 protein and DNA repair which will make it more difficult to detect DNA damage. Biomarkers that indicate activation of these pathways like for example activation of p53 might be a more sensitive method to detect genotoxic potential in the HepG2 cells.

Testing of 16 additional compounds

Testing of an additional set of 16 genotoxic compounds confirmed results that were obtained with the ECVAM compound list. The sensitivity of the CHO-k1 cell line was with 69% (11/16) higher than the sensitivity of 56% (9/16) that was obtained for the HepG2 cell line. Combining the genotoxic compounds in both compound sets results in a sensitivity of 75% (27/36) for the CHO-k1 cell line and of 58% (21/36) for the HepG2 cell line.

Discrimination of aneugens from clastogens based on size-classification

The standard method to make a discrimination between clastogens and aneugens in the micronucleus is by making use of CREST-staining or in situ hybritization [19-22]. These two staining techniques both visualize the centromeric region of the chromosome. With clastogenic compounds, a lower percentage (approximately 2-23%) of centromere positive micronuclei is found as compared to aneugens (approximately 74-94%) [21]. In addition, telomeric probes can be used to differentiate between whole chromosomes formed by aneugenic compounds and centric chromosome fragments formed by clastogenic compounds [23]. These standard methods are in principle applicable in a

HCS format however these methods are laborious, as additional staining, imaging (100x up to a 1000x magnification), and analysis is needed [22, 23].

A method that is more transferable into a high-throughput format is the recently published method by Hashimoto et al. [14] which uses size-discrimination of micronuclei to classify between clastogens and aneugens. This method was in the present study modified into a high content screening method.

Of the 32 compounds that were genotoxic in the CHO-k1 cell line, seven showed an aneugenic effect in the CHO-k1 cell line, i.e. propyl gallate, taxol, 17β-estradiol, 2-methoxy-estradiol, 2,4-dichlorophenol, 17β-ethinylestradiol, and chloramphenicol. For the natural estrogens 17β-estradiol and 2-methoxy-estradiol it has been reported that these compounds are aneugenic [24]. The synthetic estrogen 17β-ethinylestradiol gave a percentage of type II MN that was just higher than that of 2-methoxy-estradiol. 4-Hydroxy-estrone was also reported to cause microtubule disruption however this compound was 20-times less active than 2-methoxy-estradiol [24]. In the present study 4-hydroxy-estrone was classified as clastogenic; induction of DNA strand breaks has been reported for 4-hydroxy-estrone [25]. For chloramphenicol, 2,4-dichlorophenol and propyl gallate the data about clastogenicity or aneugenicity is very limited. For propyl gallate and chloramphicol weak genotoxic effects causing DNA fragmentation were reported [26, 27]. For the 2, 4-dichlorophenol no such data are available. The topoisomerase II inhibitors doxorubicin, etoposide, and ellipticin that were classified as clastogens in this study, can show clastogenic as well as aneugenic effects, however clastogenic effects seem to be the most prominent [28, 29]. Sodium arsenite which had a median that was just below the threshold of 30%, has been reported to be a clastogenic compounds [13]. For the remaining compounds the mode of action is probably indeed clastogenic [13].

The usage of a liver-S9 metabolic fraction in CHO-k1 and HepG2 cells

Like in the regulatory IVMN assay (draft guideline) a S9-liver metabolic fraction was used to mimic metabolism in the CHO-k1 HCS IVMN assay. This metabolic fraction was able to activate most proximate genotoxins, i.e. cyclophosphamide (CYP2B6), 2-acetylaminofluorene (CYP1A2), IQ (N-acetyl transferases), PhIP.HCl (N-acetyl transferases).

The proximate genotoxins benzo[a]pyrene and DMBA, which are activated by CYP1A1 and/or CYP1B1 [13], clearly showed activity without S9 mixture in CHO-k1 cells, which suggests that these CYPs are present in the CHO-k1 cells. Literature about CYP1A1/1B1 activity in CHO-k1 cells is not available; however the aryl hydrocarbon receptor that activates these enzymes is functionally active in the CHO-k1 cell line [30]. The presence of S9 mixture even resulted in a decrease of the genotoxic effects of B[a]P and DMBA, indicating that formation of reactive metabolites within the cell is

more effective than formation outside the cell.

Three compounds which in principle do not need metabolic activation, i.e. p-chloroaniline, azidothymidine, and chloramphenicol [13], showed higher genotoxic effects in the presence than absence of S9 mixture. Here a decrease of cytotoxicity resulted in more prominent genotoxic effects.

HepG2 cells were in principle only tested without the presence of S9 mixture as a certain level of metabolism is present in these cells. However, to explain differences between the two cell lines we tested all compounds that were activated by S9 mixture in CHO-k1 cells but lacked activity in HepG2, in HepG2 cells in the presence of S9. The protocol gave satisfactory results for this cell line as we clearly could activate the genotoxicity of aflatoxin B1, etoposide and progesterone. Like in the CHO-k1 cell line, two different modes of action activated the compounds. In case of aflatoxin B1 it was metabolic activation. In case of progesterone and etoposide application of S9 mixture resulted in lower cytotoxicity which made it possible for the genotoxic effect to appear. For another nine compounds no effect of the addition of S9 mixture was observed.

In summary, two automated image analysis assisted HCS in vitro micronucleus assays were developed and validated by using 62 reference compounds from an ECVAM compound list and an additional set of 16 genotoxic compounds. The predictivity of both assays for in vivo genotoxicity was satisfactory and in the same order of magnitude. In addition, the two developed assays in CHO-k1 and HepG2 cells allow differentiation of genotoxins in clastogens and aneugens. Application of these assays in the early discovery phase of drug development may proof to be a useful strategy to early assess genotoxic potential.

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Chapter 6

The identification of biomarkers for genotoxicity in HepG2 cells by gene expression profiling

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Abstract

In the present study gene expression profiling in HepG2 cells was used to find biomarkers (genes and responsive elements) that can be used for the development of high-throughput luciferase based reporter assays that can assess the genotoxic potential of compounds and discriminate genotoxic carcinogens from non-genotoxic liver toxicants.

HepG2 cells were exposed to cytotoxic concentrations of the non-genotoxic liver toxicants as cytotoxicity can activate stress pathways, may cause DNA damage, and may thus result in a false-positive in vitro prediction for in vivo genotoxicity. The differences between the expression profiles of the genotoxic and liver non-genotoxic toxicants were used to determine biomarker genes or responsive elements that are specific for genotoxins. Application of such biomarkers may result in a high-throughput luciferase based assay with a good sensitivity for genotoxicity and low false positive rate for non-carcinogens and in vivo genotoxicity.

HepG2 cells were treated with four genotoxic and seven non-genotoxic liver toxicants for 6h, 24h and 72h. Discrimination between the two classes of compounds was limited when all differentially expresses genes (DEG) were used. Pathway analysis of the DEG in the class of genotoxins revealed multiple affected pathways, of which the main ones at the three time points were involved in cell cycle control. At 72h several of the genotoxicity pathways were also activated by the non-genotoxic liver toxicants (i.e. p53 pathway, ATM pathway, G2/M cell cycle checkpoint regulation).

Additional data analysis with Qlucore Omics Explorer was performed to find genes that differentiate both classes of compounds. This analysis yielded for each time point a subset of genes (6h, 362 genes; 24h, 1914 genes; 72h, 498 genes). Pathway analysis showed that the 6h and 24h gene subsets were mainly involved in the cell cycle, apoptosis, and DNA repair. No discriminating pathways were revealed by the 72h subset of genes.

The most pronounced induced genes (top 10) in the three sets of genes were mainly downstream targets of p53, involved in apoptosis or the oxidative stress response. Several other pivotal genes involved in the DNA damage response were also present in the three subsets (i.e. GADD45A, GADD45B, PCNA, POLH, and XPC).

In conclusion, gene expression analysis with Qlucore Omics Explorer revealed a 6h, 24h, and 72h gene subset that discriminated genotoxic from non-genotoxic toxicants exerting their effect through cytotoxicity. The most prominent induced genes in the three subsets of genes were mainly involved in cell cycle control and downstream targets of p53. Further studies are undergoing to assess the role of the individual gene expression in luciferase based reporter assays which may provide a tool for screening genotoxicity with lower levels of false positives.

Introduction

In drug development toxicity is an important factor for drug attrition [1]. Genotoxicity is one of the important causes of toxicity [2]. Toxicity screening in the early discovery phase, requires the use of assays that have a high-throughput with the need for a low amount of compound [2, 3]. In this respect luciferase based reporter assays in bacteria, yeast, and (human) cell lines have been proposed as useful model systems to early assess genotoxic potential.

We have explored the bacterial based luciferase reporter assay Vitotox™ assay as pre-screen for bacterial mutagenicity [4]. The Vitotox™ appeared to be a valid high-throughput pre-screen for the Ames assay. In addition the yeast based reporter assay RadarScreen was explored as assay to detect chromosome damage [4, 5]. The RadarScreen assay showed a high sensitivity for genotoxicity, however also a high false positive rate for non-carcinogens and in vivo genotoxicity. A high false positive rate for non-carcinogens is also observed with the regulatory in vitro mammalian genotoxicity assays [6]. Factors that may play a role in the high false positive rate of currently applied mammalian cell lines are their lack of phase I and II metabolism, the absence of an active Nrf2 electrophile responsive system or functionally active p53 protein and their lack of active DNA repair [7-11]. Not only the rodent cell lines used in regulatory genotoxicity assays but also the recently validated yeast strain (RadarScreen) generally are deficient in one or more of these properties [7-10]. As HepG2 cells contain these properties [5, 11-13], the development of luciferase based assays in the HepG2 cell line may result in a high-throughput assay with an improved predictivity for genotoxicity (viz. good sensitivity and low rate of false positives).

In the present study gene expression profiling in HepG2 cells was used to assess genes or responsive elements that can be used for the development of luciferase based reporter assays to detect genotoxic potential at a low false positive rate.

HepG2 cells have been successfully used to discriminate genotoxic from non-genotoxic carcinogens by gene expression profiling [14-16]. Classifiers that discriminated genotoxic and non-genotoxic carcinogens were involved in cell cycle regulation, cell cycle arrest, DNA damage related processes, immune and stress responses, and apoptosis [14-16]. Expression profiling in HepG2 cells showed that genotoxic and non-genotoxic carcinogens could be discriminated at a relative short exposure period (12h) and at longer exposure periods (24h and 48h). Additional studies in primary mouse hepatocytes have shown that the difference between the expression profiles of genotoxic- and non-genotoxic compounds becomes more apparent in time, which improved the classification [17]. The focus in these studies was on the determination of genotoxic and non-genotoxic pathways involved in carcinogenesis and therefore in these studies non-

toxic concentrations of the non-genotoxic carcinogens were used.

In the present study gene expression profiling in HepG2 cells was used to find genes and responsive elements that can not only assess the genotoxic potential of compounds but also discriminate genotoxic carcinogens from non-genotoxic liver toxicants. HepG2 cells were exposed to the non-genotoxic liver toxicants at concentrations that give a clear cytotoxic response. Cytotoxic concentration of the non-genotoxic liver toxicants were used since cytotoxicity can activate stress pathways, may cause DNA damage, and may thus result in a false-positive in vitro prediction for non-carcinogens or in vivo genotoxicity [8]. The selection of genes and responsive elements that are specific for genotoxic carcinogens and are thus not activated by non-genotoxic liver toxicants might identify genes or response elements to use in the development of a luciferase based reporter assay with a good sensitivity and low false positive rate.

HepG2 cells were treated with four well known genotoxic carcinogens and seven non-genotoxic toxicants. The genotoxic carcinogens used in the present study were the topoisomerase II inhibitor doxorubicin [18, 19], the topoisomerase I inhibitor camptothecin [19-21], the DNA crosslinker cisplatin [22], and the adduct former benzo[a]pyrene (B[a]P). B[a]P is a proximate genotoxin that is activated by cytochrome P450 (CYP) 1A and epoxide hydrolase 1 [23]. Moreover, B[a]P activates the aryl hydrocarbon receptor [24]. The non-genotoxic liver toxicants used were the necrotic compounds paracetamol (APAP, acetaminophen) [25], carbon tetrachloride (CCl_4) [26] and iproniazid [25]. The two liver cholestatic compounds used were chlorpromazine and α-naphthylisothiocyanate (ANIT) [25]. Subsequently gliotoxin, a compound causing apoptosis [27], and TNFα, an inducer of inflammation were tested.

HepG2 cells were treated with the compounds for 6h, 24h, and 72h to be able to study the level of discrimination between the genotoxic and non-genotoxic toxicants in time and to study the activation of typical pathways involved in genotoxic responses for both the genotoxins compared to the non-genotoxic toxicants in time.

Materials and methods

HepG2 cell culture

HepG2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured in Dulbecco's Modified Eagles medium and Nutrient mixture F-12 (In Vitro Technologies Inc., Baltimore, USA) mixed in a ratio of 1:1 with 10% defined supplemented bovine calf serum from Hyclone (Utah, USA), 1% Penicillin-Streptomycin (10,000 U/ml, Gibco), and 0.05% of an aqueous mixture containing ethanolamine (2.44 ml/l), sodium selenite (0.9 mg/l) and 2-mercaptoethanol

(4.2 ml/l). Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37 °C and medium was refreshed every three or four days with sub-culturing.

Compound Treatment

HepG2 cells were seeded in 9 cm Petri dishes in a density of $5x10^5$ cells/ml in a total volume of 10 ml. Cells were cultured overnight at 37º C and a humidified atmosphere with 5% CO_2. Then medium was replaced by medium containing the genotoxic or non-genotoxic compounds. All compounds were of analytical grade and obtained from Sigma-Aldrich (St. Louis, Missouri, USA). Compounds were dissolved in 100% dimethyl sulfoxide (DMSO). DMSO solutions were added to culture medium leading to a final DMSO concentration of 1%. The concentrations of the genotoxins used were based on genotoxic concentrations in the Vitotox™ and or RadarScreen assay [4]. The non-genotoxins were tested at concentrations causing 20%-50% cytotoxicity [28] with the exception of TNFα, which was tested at a concentration giving a maximum induction of the caspase-3 activity in HepG2 cells (based on unpublished MSD data).

RNA isolation and quality control

After exposure, total cellular RNA was isolated. To this end, cell culture medium was removed and cells were washed once with 10 ml PBS (without Ca^{2+}/Mg^{2+}). Then PBS was removed and 1 ml of Trizol Reagent (Invitrogen) was added to the Petri dishes. After a short incubation period of 1 minute, Trizol reagent containing cell lysate was collected in a 2 ml Eppendorf tube and mixed. Then 200 µl chloroform was added and the tubes were shaken vigorously by hand. Samples were centrifuged for 15 min at 12,000*g* at 4°C. The upper aqueous phase was transferred to a new Eppendorf vial and placed on ice. Of the supernatant, 200 µl was used for further purification steps. The remaining supernatant was stored in a freezer at -80 ºC. Then 200 µl of 70 % ethanol were added to an equal volume of supernatant. After mixing, the sample was loaded on a Purelink™ Micro-to-Midi total RNA Purification System column (Invitrogen). RNA was purified according to the manufacturer's protocol. RNA was eluted by using 30 µl of RNAse free water.

The RNA concentration and quality of the samples was determined with use of the nanodrop ND-1000 spectrophotometer and bioanalyzer 2100 (Agilent Technologies, Amstelveen, The Netherlands). The OD260/OD280 values of all samples were >1.8 and the RNA integrity values [29] were >9.

RNA processing, DNA hybridization and staining of the arrays

The GeneChip 3'IVT Express kit (Affymetrix, Santa Clara, CA) was used to generate biotinylated DNA from the RNA template. Labelled cDNA was fragmented, checked on the Bioanalyzer 2100 and hybridization mixtures were prepared (Affymetrix

hybridization wash and stain kit). Then 300 µl 50 ng/µl labeled DNA were hybridized at 45 ºC for 16-17 h to the Affymetrix GeneChip HGU133_Plus_2. Gene chips were washed and stained by using an Affymetrix fluidics station 450. The arrays were laser scanned with a GeneChip scanner 3000 (Affymetrix). All steps were performed according the manufacturer's instructions.

Data processing and gene expression analysis

First, the complete set of data was processed and analyzed. Second, Qlucore Omics Explorer 2.1 (Qlucore AB, Lund, Sweden) was used to assess subsets of genes that discriminated the genotoxic from non-genotoxic toxicants.

Affymetrix CEL files were processed in Rosetta Resolver (v7.2, buid 7.2.2.0.0). Intensity Profiles were built using the Affymetrix Rosetta-Intensity Profile Builder using PM and MM probes. Affymetrix Rosetta-Intensity Experiment Builder combines the expression values for individual profiles and normalizes the data. Ratios were calculated from simple arithmetic averages of numerator (compound treated samples) and denominator (vehicle treated samples). The corresponding p-values were calculated using a two-sided two-sample *t* test. Probesets that did not produce significant ratios ($p<0.05$) in any contrast were removed from the dataset.

Subsequent data analysis was performed on $\log_{10}$ratios in Qlucore Omics Explorer 2.1 (Qlucore AB, Lund, Sweden). At each of the time points 6h, 24h and 72h a t-test identified the significantly discriminating genes ($p \leq 0.01$) between samples from genotoxic and non-genotoxic toxicants.

CoPub and Ingenuity Pathway Analysis

The differentially expressed genes (>2-fold, $p<0.05$) were analyzed by using Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com). Ingenuity pathway analysis was performed to identify the pathways from the Ingenuity Pathways Analysis library of canonical pathways that were most significant to the data set ($p<0.001$).

In addition, the three time-related gene sets (6h, 24, 72h subset) acquired with Qlucore Omics Explorer 2.1 were analyzed ($p<0.001$) through the use of CoPub (www.copub.org) [30] and Ingenuity Pathways Analysis (IPA). CoPub was used to link genes to biomedical pathways. CoPub uses thesaurus-based keyword matching in Medline abstracts to link genes to pathways [30].

Results

1. Analysis of gene expression profiles by using the complete gene set

Analysis of the expression profiles, revealed that 22,243 genes were significantly regulated ($p<0.05$) in at least one of the treatments at the three time points. The number of differentially expressed genes (DEG) per compound and time point are summarized in Table 1 (>2-fold up- or down-regulation, $p<0.05$). All compound treatments were effective as they caused a significant number of expression changes at 6h, 24h, and 72h.

Table 1. The number of differentially expressed genes (DEG) per compound treatment and time point. The threshold for regulation was set at 2-fold ($p<0.05$).

Compound	Concentration (mol/l)	Time point (h)	Total number of DEG	Up-regulated DEG	Down-regulated DEG
Genotoxins					
Benzo[a]pyrene	$1.00x10^{-4}$	6	7681	1571	6110
		24	3880	1426	2454
Camptothecin	$1.00x10^{-6}$	6	5451	1672	3779
		24	7536	2889	4647
		72	6547	2809	3738
Cisplatin	$3.16x10^{-4}$	6	1429	1045	384
		24	1076	584	492
		72	1062	641	421
Doxorubicin	$3.16x10^{-7}$	6	1253	927	326
		24	3069	1374	1695
		72	4071	2407	1664
Non-genotoxic liver toxicants					
ANIT	$1.00x10^{-6}$	6	219	91	128
		24	246	133	113
		72	1726	151	1575
APAP	$1.00x10^{-3}$	6	320	86	234
		24	302	144	158
CCl_4	$1.00x10^{-3}$	6	203	79	124
		24	365	86	279
Chlorpromazine	$1.00x10^{-5}$	6	212	118	94
		24	323	187	136
		72	2924	2312	612
Gliotoxin	$1.00x10^{-6}$	6	511	164	347
		24	491	169	322
		72	1829	1388	441
Iproniazid	$1.00x10^{-3}$	6	234	79	155
		24	292	88	204
TNF-α	50 ng/ml	6	415	196	219
		24	565	373	192
		72	3778	2700	1078

Figure 1 shows the total number of DEG for the class of genotoxins and non-genotoxic toxicants at 6h, 24h, and 72h. Treatment with the genotoxins gave more pronounced expression changes at all time points compared to the non-genotoxic toxicants. The total number of DEG was already maximal at 6h and declined thereafter. The number of DEG increased in time for the class of non-genotoxic toxicants amounting to 6541 DEG at 72h.

The overlap in time of the DEG at 6h, 24h and 72h was analyzed for both classes of compounds and is shown in a Venn diagram (Figure 2). The overlap between the DEG in the class of genotoxins at the three time points was substantial. Of all 11739 DEG at 6h, 47% (5526 genes) were also present at 24h. Of all 10310 DEG at 24h, 57% (5842 genes) were also present at 72h.

The overlap in time between the DEG in the class of non-genotoxic toxicants was (significantly) less prominent compared to the genotoxins. Of all 1656 DEG at 6h, 31% (514 genes) were also present at 24h. Subsequently, of all 1978 DEG at 24h, 38% (759 genes) were also differentially expressed at 72h.

For the four genotoxins, significantly altered pathways were assessed with ingenuity pathway analysis (IPA). Multiple pathways were significantly affected by the genotoxic compounds at 6h, 24h and 72h (Table 2). For each genotoxic compound there were some unique altered pathways, but there were also shared pathways. The p53 pathway was significantly affected by all four genotoxins. In addition four other pathways in which p53 and MDM2 play an pivotal role were affected by two or three genotoxins, i.e. the 'molecular mechanisms of cancer pathway', the ATM pathway, the cell cycle: G1/S checkpoint regulation pathway, and the cell cycle: G2/M DNA damage regulation pathway.

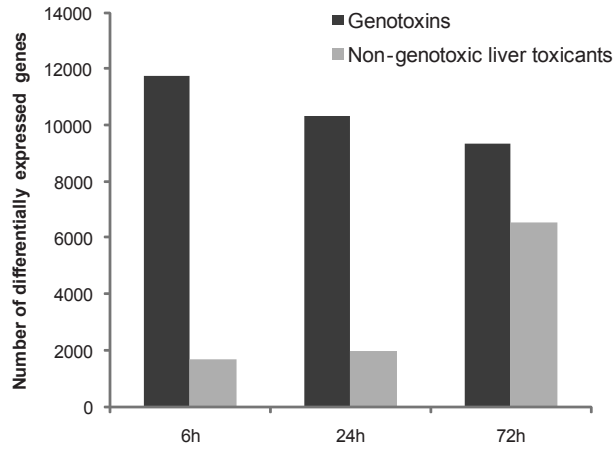

Figure 1. Total number of differentially expressed genes in the compound class of genotoxins and non-genotoxic liver toxicants at 6h, 24h and 72h (see results section 1).

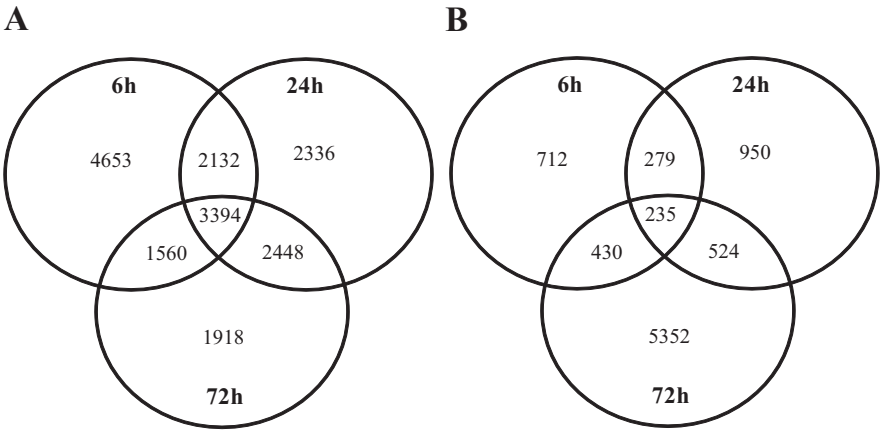

Figure 2. Venn diagram showing the overlap of the differentially expressed genes at 6h, 24h, and 72h for the compound class of (A) genotoxins and (B) non-genotoxic liver toxicants (see results section 1).

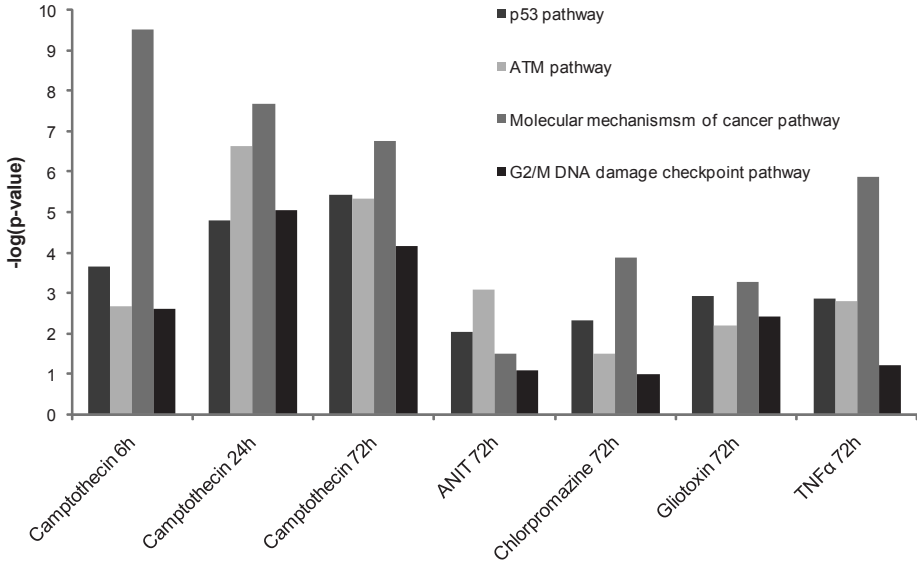

Figure 3. The activation of four pathways involved in the DNA damage response for the topoisomerase I inhibitor camptothecin at 6h, 24h, and 72h and for four non-genotoxic liver toxicants at 72h (see results section 1).

The activation of these pathways was also analyzed for the seven non-genotoxic toxicants. None of the non-genotoxic toxicants activated these pathways at 6h and 24h. However, activation of these pathways was observed at 72h which indicates a correlation between activation of these pathways and induced cytotoxicity. The results are shown in Figure 3, which also shows the activation of these pathways by camptothecin, which

was one of the four genotoxic carcinogens used in the present study. The activation of the pathways by the non-genotoxic liver toxicants was less prominent compared to activation by camptothecin.

The overlap of DEG between the two compound classes was also analyzed (Venn diagram not shown). Of all 1656 DEG in the class of non-genotoxic liver toxicants at 6h, 60% (986 genes) were also present in the 6h set of DEG for the class of genotoxins. These values were 66% (1309 genes) for 24h and 52% (3388 genes) for 72h. This analysis clearly shows that many of the DEG in the class of non-genotoxic liver toxicants, were also DEG in the class of genotoxins, pointing at DEG that do not provide good candidate biomarkers for discriminating between the two classes on the basis of DEG. On the other hand there are many DEG specific for the class of genotoxins.

Table 2. Summary of the significantly affected pathways assessed with IPA ($p<0.001$) per genotoxic compound and time point. A maximum of ten pathways are shown. The most significantly affected pathways are shown first.

Compound	Time point (h)	Significantly affected pathways
Benzo[a]pyrene	6	Protein ubiquitination pathway, role of BRCA1 in DNA damage response, hereditary breast cancer signaling, JAK/Stat signaling, estrogen receptor signaling, glucocorticoid receptor signaling
	24	p53 Signaling, AhR signaling, molecular mechanisms of cancer pathway, cell cycle: G1/S checkpoint regulation
Camptothecin	6	Molecular mechanisms of cancer pathway, prolactin signaling, RAR activation, estrogen receptor signaling, glucocorticoid receptor signaling, TGF-β signaling, PDGF signaling, factors promoting cardiogenesis in vertebrates, B cell receptor signaling, ERK5 signaling
	24	Molecular mechanisms of cancer pathway, role of BRCA1 in DNA damage response, ATM signaling, role of CHK proteins in cell cycle checkpoint control, cell cycle: G2/M DNA damage checkpoint regulation, mitotic roles of polo-like kinase, p53 signaling, protein ubiquitination pathway, hereditary breast cancer signaling, cell cycle: G1/S checkpoint regulation
	72	Molecular mechanisms of cancer pathway, p53 signaling, ATM signaling, cell cycle: G2/M DNA damage checkpoint regulation, mitotic roles of polo-like kinase
Cisplatin	6	p53 Signaling
	24	TR/RXR activation
	72	TR/RXR activation, LXR/RXR activation, biosynthesis of steroids
Doxorubicin	6	Cell cycle: G2/M DNA damage checkpoint regulation
	24	p53 Signaling, AhR signaling
	72	Hereditary breast cancer signaling, role of BRCA1 in DNA damage response, p53 signaling, role of CHK proteins in cell cycle checkpoint control, molecular mechanisms of cancer, pyrimidine metabolism, AhR signaling, ATM signaling, purine metabolism, renal cell carcinoma signaling

Principal component analysis (PCA) was performed to study the discrimination of the two compound classes (Figure 4A). PCA shows that it is not possible to discriminate the class of genotoxic and non-genotoxic toxicants at the 6h time point. At 24h and 72h it was possible to slightly distinguish the two compound classes. The limited discrimination between the two compound classes that was observed with the PCA is probably caused by the substantial overlap of DEG between the two compound classes at the three time points.

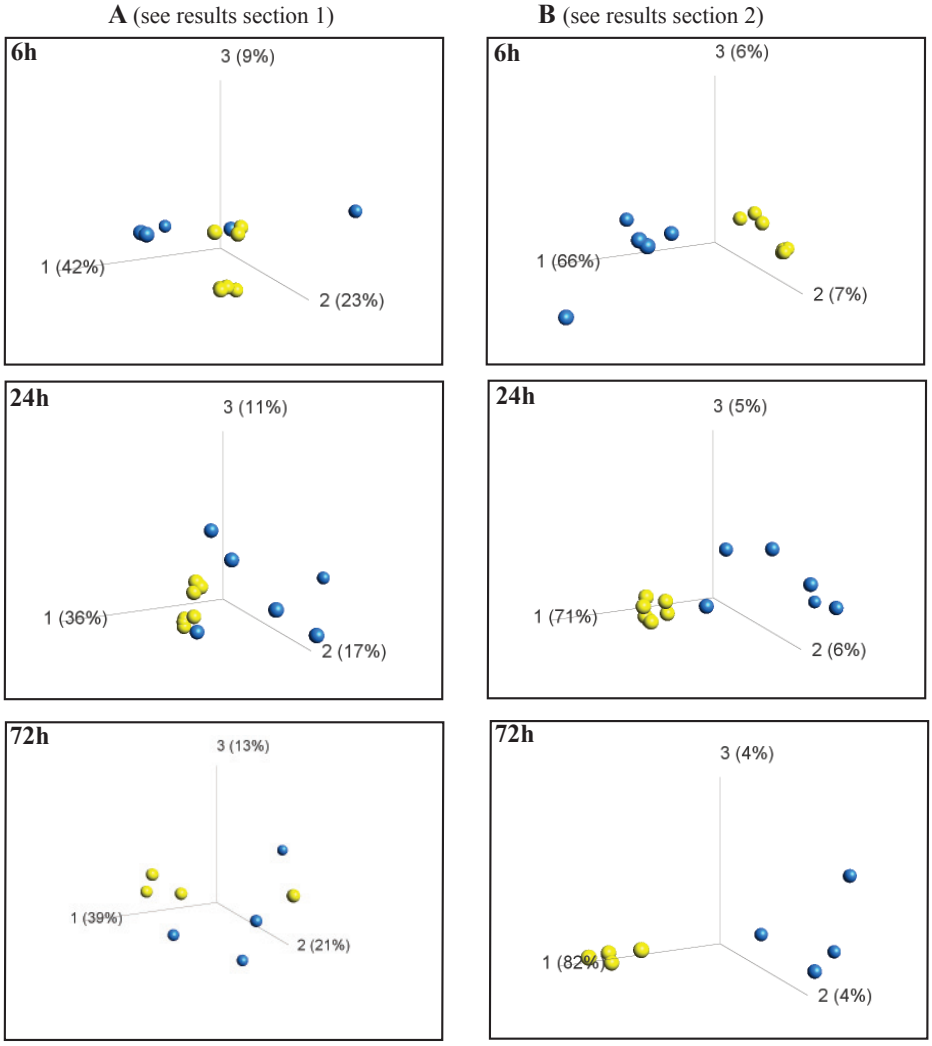

Figure 4. PCA pictures showing the discrimination of the genotoxins (blue color) and non-genotoxic liver toxicants (yellow) by using all differentially expressed genes (A) and the gene subsets calculated by Qlucore (B) at the three time points.

Pathway analysis of the complete set of DEG indicated that pathways involved in cell cycle control may contain genes that can be used as biomarkers as these pathways were significantly activated by the four genotoxicants tested. However, not all genes in these pathways seem to be good biomarkers as these pathways were moderately activated by the non-genotoxic toxicants at 72h. Additional analysis of the gene expression profiles was performed to find a specific set of DEG that can discriminate the two classes of compounds at the three time points.

2. Analysis of gene expression profiles by using Qlucore Omics Explorer

2.1 The identification of potential biomarker genes

The complete set of 22,243 DEG was further analyzed by using Qlucore Omics Explorer 2.1 to identify potential biomarker genes that discriminate the genotoxic from the non-genotoxic toxicants. For each time point, a subset of genes containing potential biomarkers was generated (6h, 362 genes; 24h, 1914 genes; 72h, 498 genes). The three subsets of genes are specified in detail in Table 3, which shows the number of genes, the relative expression of the genes, and the difference in fold induction per gene between the two compound classes. The mean induction per class was used for the calculations.

The overlap between the three subsets was limited and is shown in a Venn diagram (Figure 5). The 6h-24h and 24h-72h sets of genes, have an overlap of 52 and 81 genes, respectively. Only 15 genes were present in both the 6h and 72h subset and four genes were present at all three time points.

These four genes were cyclin-dependent kinase inhibitor 1A (CDKN1A, p21), MDM2, MDM4, and diacylglycerol kinase, which are all downstream targets of p53 [31]. The CDKN1A protein has multiple functions. CDKN1A is a potent cyclin-dependent kinase inhibitor. CDKN1A binds to and inhibits the activity of cyclin-CDK2 or -CDK4 complexes, and functions as a regulator of cell cycle progression at G1. This protein also regulates proliferating cell nuclear antigen (PCNA), which has a regulatory role in DNA repair and S phase DNA replication [32, 33]. PCNA is present in the 72h set of biomarkers. MDM2 is a nuclear phosphoprotein that binds and inhibits transactivation by tumor protein p53, as part of an autoregulatory negative feedback loop. MDM4 plays a role in stabilizing p53 and thereby protects p53 from MDM2 mediated degradation [34]. Diacylglycerol kinase is a kinase that competes with protein kinase C for diacylglycerol.

A PCA by using the 6h, 24h and 72h subsets of genes that were acquired with Qlucore Omics Explorer is shown in Figure 4B. At each time point there is a clear discrimination between the two compound classes. Although the total number of genes in the 72h gene subset was less than at 24h, the difference between the two compound classes in the PCA was larger. This is due to the larger difference in expression between the two compound classes at 72 h.

Table 3. Specification of the 6h, 24h, and 72h gene subsets assessed with Qlucore Omics Explorer 2.1.

Qlucore gene subset	Total number of genes	Genes with higher expression in the class of genotoxins		Genes with lower expression in the class of genotoxins	
		Total number	Difference in fold induction per gene	Total number	Difference in fold induction per gene
6h	362	189	0.87	173	-0.41
24h	1914	913	0.98	1001	-0.34
72h	498	240	0.80	248	-0.65

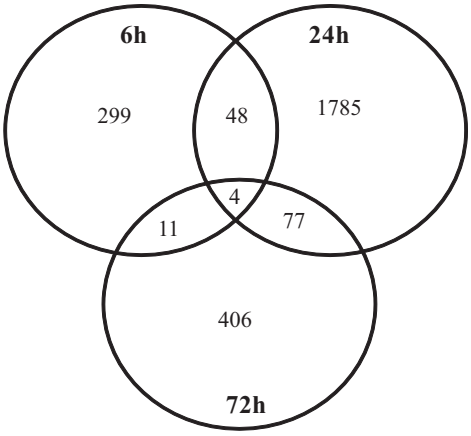

Figure 5. Venn diagram showing the overlap of the 6h, 24h, and 72h subsets of genes that were assessed with expression analysis using Qlucore Omics Explorer 2.1 (see results section 2.1).

2.2 Further analysis of the most prominent induced genes

After Qlucore analysis, the most prominent induced genes in the class of genotoxins at 6h, 24h, and 72h may provide the most promising leads for the generation of luciferase based reporter assays that can detect genotoxic potential. These most prominent induced genes (top 10) were analyzed in detail (Table 4; abbreviations of the genes are also clarified in this table).

In the 6h subset, ATF3 (3.22 fold) showed the highest fold induction. ATF3 is a transcription regulator that acts like a stress sensor which activates p53 by blocking its ubiquitination [35, 36]. PLK2, CDKN1A, MDM2, and BTG2 are downstream target genes of p53. As described earlier CDKN1A and MDM2 have pivotal roles in the p53 response. PLK2 and BTG2 have a role in cell cycle checkpoints [37, 38]. Of the remaining 5 genes, SERTAD1 and DUSP1 (MKP1) have a role in cell cycle regulation (p53 independent) [39, 40]. PVRL4 encodes an adhesion receptor that functions in cell-cell adhesion and has been proposed as a diagnostic and therapeutic target for lung cancer [41]. In addition, induction of GABRE was observed in the class of genotoxicants. GABRE is coding for an ion channel protein that is activated by several classes of drugs including genotoxins [42]. ICOSLG is a co-stimulator ligand that increases the

production of cytokines [43]. With exception of ICOSLG the most prominent induced genes in the 6h subset were also present in one or both of the 24h and 72h subsets.

In the 24h subset, RRAD showed the most pronounced induction. RRAD is a GTPase that plays a role in carcinogenesis. This gene is silenced by methylation in a variety of cancers like prostate, lung, breast and cervical carcinoma [44-46]. Regulation by the p53 downstream target stratifin has been reported [47]. The p53 downstream targets CEL, PLK3 and TP53I3 were also present in the 24h set of biomarkers. CEL has a physiological role in cholesterol and lipid-soluble vitamin ester hydrolysis and absorption. The link with genotoxicity is presently unknown. However, CEL has been reported to be a p53 downstream target [48]. PLK3 and TP53I3 have a function in the response to oxidative stress [49]. For the remaining six genes no link with p53 has been reported. RASD1 is a suppressor of cell growth [50], and CSTA is an anti-apoptotic protein [51, 52], CYGB is involved in the oxidative stress response [53] and MR1 has a role in the immune response (antigen presentation). CABYR is a calcium binding tyrosine phosphorylation regulated fibrous sheath protein that is an antigen that is specifically expressed in certain tumors and has therefore been proposed as a promising target for immunotherapy for lung cancer patients [54]. PVRL4 was, similar to the 6h set, one of the top 10 induced genes in the 24h subset. CEL, PLK3, CYGB, MR1 were also present in another subset. The average fold changes were higher at 24h and 72h compared to 6h for the top 10 induced genes.

In the 72h subset, CEL was the gene with the highest induction. Other p53 downstream targets present in the 72h subset were SULF2 [55], MDM2 and DDB2. DDB2 participates in nucleotide excision repair [56]. Of the remaining six genes, PDE4C has a role in regulation of apoptosis and the cell cycle and FDXR and CYGB have a role in the oxidative stress response [49]. APL1 is involved in L-methionine salvage [57], BMP8A [58] is a growth factor and ELOVL3 has a function in fatty acid chain elongation [59]. To illustrate the specificity of the biomarker genes box plots are given for CEL, MDM2 and CSTA (Figure 6). Five of the most pronounced genes at 72h were also present in another subset.

Table 4. Expression changes of the most prominent induced genes (top 10) in the class of genotoxins for the 6h, 24h and 72h subsets of genes acquired with Qlucore Omics Explorer 2.1. The mean expression in the two classes of compounds is shown. Numbers in bold indicate expression changes higher than 1.5 fold and cells with a grey background indicate the presence of the gene in the gene subset at that time point.

Gene subset	No	Gene symbol	Gene Name	Mean induction genotoxic toxicants			Mean induction non-genotoxic liver toxicants		
				6h	24h	72h	6h	24h	72h
6h	1	ATF3	activating transcription factor 3	**3.22**	**4.13**	0.59	1.07	0.91	1.16
	2	PLK2	polo-like kinase 2 (Drosophila)	**3.16**	**5.33**	**3.31**	0.87	0.86	1.03
	3	SERTAD1	SERTA domain containing 1	**3.13**	**4.37**	**2.02**	1.12	0.98	0.81
	4	CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	**2.94**	**5.84**	**3.50**	0.99	1.20	0.79
	5	MDM2	MDM2 p53 binding protein homolog (mouse)	**2.80**	**3.78**	**5.11**	1.01	1.07	1.28
	6	PVRL4	poliovirus receptor-related 4	**2.73**	**6.17**	**4.58**	0.98	0.90	1.13
	7	DUSP1	dual specificity phosphatase 1	**2.46**	**3.46**	0.89	0.74	1.31	**2.03**
	8	GABRE	gamma-aminobutyric acid (GABA) A receptor, epsilon	**2.45**	**2.24**	**1.54**	0.80	0.85	0.81
	9	ICOSLG	inducible T-cell co-stimulator ligand	**2.38**	**2.00**	0.70	0.84	1.08	0.77
	10	BTG2	BTG family, member 2	**2.36**	**4.02**	**2.40**	0.86	1.02	0.83
24h	11	RRAD	Ras-related associated with diabetes	**9.39**	**19.32**	**5.28**	**1.65**	1.14	0.99
	12	CEL	carboxyl ester lipase (bile salt-stimulated lipase)	**2.67**	**16.25**	**20.45**	1.05	0.85	0.83
	13	RASD1	RAS, dexamethasone-induced 1	**3.04**	**13.47**	1.48	**2.02**	**1.85**	**1.78**
	14	CSTA	cystatin A (stefin A)	**2.69**	**12.22**	**3.85**	0.84	0.92	**1.52**
	15	PLK3	polo-like kinase 3 (Drosophila)	**2.04**	**12.15**	**2.25**	0.86	**1.53**	1.17
	16	CYGB	cytoglobin	1.19	**11.96**	**3.94**	0.89	0.96	0.60
	17	MR1	major histocompatibility complex, class I-related	**1.89**	**10.49**	**5.83**	1.06	1.25	1.31
	18	TP53I3	tumor protein p53 inducible protein 3	1.19	**9.77**	**6.32**	0.98	1.21	0.94
	19	CABYR	calcium binding tyrosine-(Y)-phosphorylation regulated	**1.58**	**6.49**	**4.47**	1.03	0.89	0.76
	6	PVRL4	poliovirus receptor-related 4	**2.73**	**6.17**	**4.58**	0.98	0.90	1.13
72h	12	CEL	arboxyl ester lipase (bile salt-stimulated lipase)	**2.67**	**16.25**	**20.45**	1.05	0.85	0.83
	20	PDE4C	phosphodiesterase 4C, cAMP-specific	0.99	**8.73**	**14.86**	1.00	1.10	0.73
	21	APLP1	amyloid beta (A4) precursor-like protein 1	1.02	**6.04**	**10.02**	1.38	1.07	0.65
	22	SULF2	sulfatase 2	1.13	**3.16**	**8.17**	1.04	0.96	0.94
	23	FDXR	ferredoxin reductase	1.42	**3.95**	**5.31**	1.04	1.03	0.85
	5	MDM2	MDM2 p53 binding protein homolog (mouse)	**2.80**	**3.78**	**5.11**	1.01	1.07	1.28
	24	BMP8A	bone morphogenetic protein 8a	1.03	**3.20**	**4.69**	1.19	0.99	0.58
	25	ELOVL3	elongation of very long chain fatty acids -like 3	**2.15**	**2.12**	**4.66**	0.99	1.05	1.12
	16	CYGB	cytoglobin	**1.54**	**11.92**	**4.46**	0.96	1.01	0.61
	26	DDB2	damage-specific DNA binding protein 2, 48kDa	1.24	**2.66**	**4.27**	0.99	1.04	0.67

2.3 Further analysis of genes involved in the DNA repair response

The expression changes of six genes involved in the DNA repair response were analyzed. These genes were GADD45A, GADD45B, PCNA, POLH, RAD51C, and XPC (Table 5). Of these genes RAD51C was the only gene that was not present in one of the three Qlucore gene subsets. Box plots for GADD45A and XPC are given as illustration for the specificity of the genes (Figure 6).

The GADD45 gene encoded proteins are a group of critical signal transducers, that are involved in many cellular regulations like growth arrest, DNA repair, cell survival, and apoptosis [60, 61]. PCNA is involved in translesion synthesis (the bypass of DNA lesions) [32, 33]. POLH is involved in post replication repair and defects in this gene result in humans in a Xeroderma pigmentosum like illness with a defect in UV damage repair [62]. RAD51C is involved in double strand DNA break repair and XPC in nucleotide excision repair [63, 64]. For RAD51C, the expression changes of doxorubicin and camptothecin alone are also shown in Table 5 as these are topoisomerase inhibitors which cause double strand DNA breaks. These data indicate that RAD51C might be a good classifier for DNA double strand breaks.

Table 5. Expression changes of several genes involved in the DNA repair response for the class of genotoxic and non-genotoxic liver toxicants. Numbers in bold indicate expression changes higher than 1.5 fold and cells with a grey background indicate the presence of the gene in the gene subset at that time point.

Gene symbol	Gene name	Mean induction genotoxic toxicants			Mean induction non-genotoxic liver toxicants		
		6h	24h	72h	6h	24h	72h
GADD45A	Growth arrest and DNA-damage-inducible, alpha	**1.84**	**2.60**	**1.59**	0.99	1.03	1.21
GADD45B	Growth arrest and DNA-damage-inducible, beta	**5.96**	**3.40**	1.17	1.21	1.05	0.90
PCNA	Proliferating cell nuclear antigen	1.03	1.27	**2.38**	1.00	1.01	0.99
POLH	Polymerase (DNA directed), eta	1.58	**3.71**	**4.00**	0.96	0.93	1.08
RAD51C	RAD51 homolog C	1.25	1.45	**2.08**	0.94	0.99	**1.58**
RAD51C[a]	RAD51 homolog C	1.07	**2.02**	**3.27**	0.94	0.99	**1.58**
XPC	Xeroderma pigmentosum, complementation group C	1.14	**2.24**	**2.64**	1.14	1.07	1.00

[a]Mean fold induction of camptothecin and doxorubicin

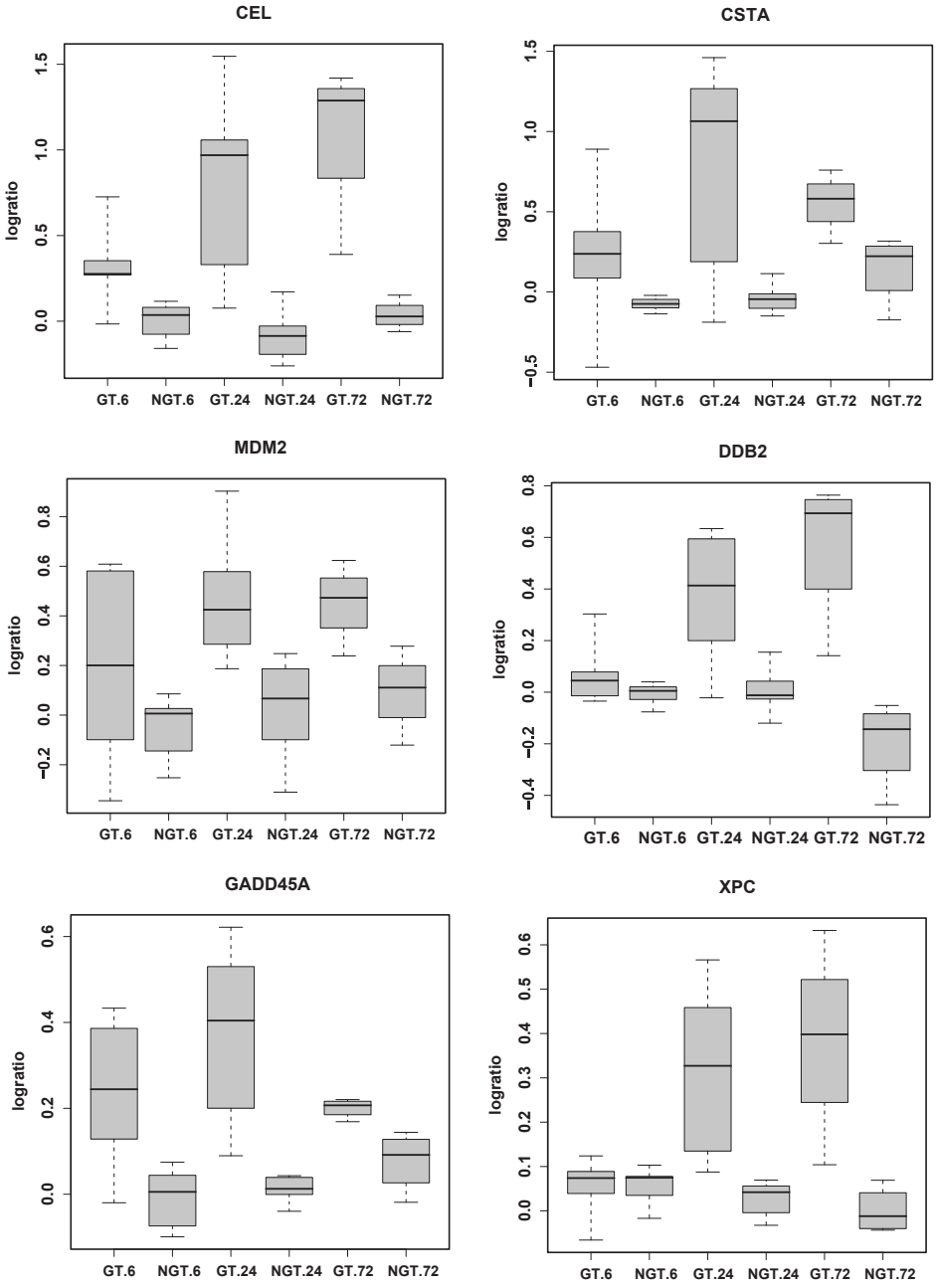

Figure 6. Boxplots showing the induction (log ratio) of six potentially useful biomarker genes, i.e. CEL, CSTA, MDM2, DDB2, GADD45A, and XPC, for the genotoxic (GT) and non-genotoxic liver toxicants (NGT) at 6h, 24h and 72h (see results sections 2.2 and 2.3)

2.4 Pathway analysis with CoPub and IPA

The three subsets (6h, 24h, 72h) of genes that were assessed with Qlucore Omics Explorer and give the best discrimination between the genotoxic and non-genotoxic toxicants were analyzed for affected pathways by using Copub and IPA.

CoPub software analysis was used to link the three subsets of genes to pathways (Table 6). At 6h, the most affected pathway by the subset of genes was the cell cycle. This was mainly caused by the set of up regulated genes in the class of genotoxins. The down regulated genes in the class of genotoxins could not be linked to a specific pathway. At 24h, the subset of genes was linked to the cell cycle, DNA replication, apoptosis, S-phase regulation of the cell cycle, protein degradation, and DNA repair. The up regulated genes in the class of genotoxins showed the strongest association with the protein degradation pathway, apoptosis, and the cell cycle regulation. The down regulated genes in the class of genotoxins were associated with DNA replication, translation, and the ribosome pathway. At 72h, no pathways were significantly affected.

IPA was also used to analyze the pathways affected by the gene three subsets (Table 7). Analysis yielded quite similar results as found with CoPub. At 6h, the affected pathways were mainly associated with regulation of the cell cycle. The p53 signaling pathway, ataxia telangiectasia mutated (ATM) signaling pathway and G2/M DNA damage checkpoint regulation were the most affected. At 24h, six pathways were affected. Like observed at 6h, the p53 and ATM signaling pathways were found to be affected. In addition 'hypoxia signaling in the cardiovascular system' was found to be affected as well as 'LPS/IL-a mediated inhibition of retinoic acid receptor (RXR) function', 'molecular mechanisms of cancer', and xenobiotic signaling. At 72h, IPA yielded in concordance with CoPub analysis no significantly affected pathways.

Table 6. Pathway analysis with CoPub ($p<0.001$) of the Qlucore Omics Explorer gene subsets.

Gene subset	**Genes up-regulated by genotoxins**	**Genes down-regulated by genotoxins**	**Complete subset of genes**
6h	Cell cycle	*None*	Cell cycle
24h	Protein degradation	DNA replication	Cell cycle
	Apoptosis	Translation	DNA replication
	Cell cycle	Ribosome	Apoptosis
			S-phase
			Protein degradation
			DNA repair
72h	*None*	*None*	*None*

Table 7. Pathway analysis of the 6h, 24h, and 72h Qlucore Omics Explorer gene subsets with IPA. Pathways affected with p<0.001 are shown.

Gene subset	Affected pathways
6h	p53 Signaling
	ATM signaling
	Cell cycle: G2/M DNA damage checkpoint regulation
24h	p53 Signaling
	Hypoxia signaling in the Cardiovascular system
	ATM signaling
	LPS/IL-a mediated inhibition of RXR function
	Molecular mechanisms of cancer
	Xenobiotic signaling
72h	*None*

Discussion

1. Analysis of gene expression profiles by using the complete gene set

The present study focused on the differences between expression profiles of HepG2 cells treated with genotoxins and cytotoxic concentrations of non-genotoxic liver toxicants. HepG2 cells were treated with four genotoxic and seven non-genotoxic liver toxicants for 6h, 24h or 72h. The responses to the genotoxins were not only more pronounced in time but also in number of DEG. Initial pathway analysis revealed that the DEG in the class of genotoxicants were mainly involved in cell cycle control.The fact that the genotoxins caused a quick response is in line with previous studies that reported that the activation of the p53 protein and DNA repair already happened 3h after DNA damage [22, 65, 66].

Based on PCA the discrimination between the two compound classes was less apparent. The moderate overlap between the DEG between the two compound classes was probably the reason. Pathway analysis indicated that pathways involved in cell cycle control might contain genes that discriminate genotoxicants from non-genotoxic toxicants as these pathways were activated by the four genotoxicants at 6h and 24h but not by the non-genotoxic toxicants. At 72h a moderate activation of these pathways was also observed for the non-genotoxic toxicants indicating that not all genes in these pathways are specific biomarkers for genotoxicity. Additional analysis was therefore needed to determine genes that discriminated both classes of compounds. For this analysis Qlucore Omics Explorer was used.

2. Analysis of gene expression profiles by using Qlucore Omics Explorer

2.1 Identification of potential biomarker genes

Qlucore Omics Explorer analysis yielded three subsets of genes that discriminated the genotoxic from the non-genotoxic liver toxicants at the three time points. At the early time point of 6h there was a set of 362 genes that distinguished the two classes. Qlucore analysis at the 24h time point yielded the highest number of potential biomarkers (1914 genes). Although the number of discriminating genes dropped at 72h, the discrimination between the genotoxic and non-genotoxic toxicants by using these 498 biomarkers was good and even better than the discrimination at 6h and 24h (Figure 4B). The activation of pathways involved in the cell cycle and DNA repair response (i.e. p53 pathway, ATM pathway, G2/M cell cycle checkpoint regulation, 'molecular mechanisms of cancer pathway') by the non-genotoxic liver toxicants contributed to the decrease in number of discriminating genes in the 72h subset.

This is in line with the discrimination between genotoxic and non-genotoxic carcinogens. Mathijs et al. [67] showed in primary mouse hepatocytes that the discrimination between genotoxic and non-genotoxic carcinogens became more apparent in time.

Although it was possible to assess three subsets of genes that clearly discriminated the genotoxic and non-genotoxic liver toxicants, the overlap between genes in the three subsets was limited. Only four genes were present in the 6h, 24h as well as the 72h subset of genes. These four genes were CDKN1A, MDM2, MDM4 and DIGK, all downstream targets of p53. The importance of the p53 pathway for classification of genotoxic compounds was later on confirmed by analysis of the most prominent induced genes (see results section 2.2) and by pathway analysis (see results section 2.4).

2.2 Analysis of the most prominent induced genes and genes involved in DNA repair

The most prominent induced genes (top 10) in the three subsets were analyzed in more detail. Many of the prominent inducers were downstream targets of p53. The remaining pronounced induced genes had mainly a role in cell cycle regulation, apoptosis or were stress sensors. More than 50% of the top 10 inducers were present in two Qlucore gene subsets. Therefore these potential biomarker genes may be useful at more than one time point.

Analysis also showed that several genes involved in the DNA repair response might be valuable biomarker genes. These genes were GADD45A, GADD45B, PCNA, POLH, and XPC. In previous studies a GADD45A reporter assay (GreenScreen HC) has been extensively validated and shows a good sensitivity and specificity for genotoxic carcinogens [68-70].

Besides general biomarkers that discriminate the genotoxic carcinogens versus non-genotoxin liver toxicants, more specific biomarkers that clarify the mechanism of genotoxins might be very useful. Topoisomerase inhibitors like doxorubicin and

camptothecin damage the DNA by the formation of double strand breaks. Such breaks are at first instance repaired by non-homologous end-joining or homologous recombination [71-72]. The RAD51 gene and homologs play an important role in double strand repair by homologous recombination. The results of the present study indicated that the RAD51C gene might be a specific biomarker for the generation of double strand breaks.

In the present study we analyzed the Fanconi genes as these enzymes are involved in repair of DNA crosslinks [73]. Activation of these genes might be valuable to detect the mode of action of DNA crosslinkers like cisplatin. However, no clear and specific activation of these genes was found in the present study. Therefore it seems that these genes are not good markers in the current HepG2 system.

2.3 Pathway analysis with CoPub and IPA

Genes in the three subsets of genes that were assessed with Qlucore Omics Explorer and discriminate the two compounds classes were analyzed with Copub and IPA. CoPub analysis showed that the genes in the 6h subset had a prominent role in the cell cycle. This was confirmed by IPA analysis which showed that the 6h subset genes were involved in the p53 pathway, ATM pathway and G2/M DNA damage checkpoint regulation.

The pathways in CoPub that were affected at the 24h time point were again mainly involved in the cell cycle. Results of CoPub suggested the occurrence of cell cycle arrest, as DNA replication, translation and ribosome pathways were down-regulated by the genotoxic compounds. Cell cycle arrest as a consequence results in more time for DNA repair. CoPub analysis indeed showed that the DNA repair pathway was activated. Because of DNA damage, the apoptosis and protein degradation pathways were probably also activated. This is confirmed by IPA analysis, where it was shown that p53, ATM, and molecular mechanisms of cancer signaling pathways were induced. In these pathways, p53, MDM2 and their downstream targets play a prominent role.

One additional pathway that was also analyzed in more detail was the IPA pathway ‘Hypoxia signaling in the cardiovascular system’. Analysis of this pathway showed a prominent role for MDM2 and p53. Activation of these genes by genotoxicity, but not by hypoxia, was likely the reason for the positive response of this pathway.

The xenobiotic signaling pathway was also affected and unexpectedly CYP3A4, 3A5 and 3A7 were significantly induced by the four genotoxic compounds. None of the genotoxins used in the present study has been reported to activate the CAR or PXR directly. The activation of the xenobiotic signaling pathway might be PXR/CAR independent which has been reported for for several other compounds [74]. In these cases there was no direct link with genotoxicity. Activation of the Nrf2 pathway might be involved in this activation [74]. Another gene in the xenobiotic signaling pathway, RXR, was approximately 2-fold down-regulated by all genotoxic compounds. Retinoid X receptors have been reported to play a pivotal role in the regulation of growth and

differentiation in normal and tumor cells. Deregulation of RXR expression has been reported in for example non-small cell lung cancer [75]. The mechanism underlying the impaired expression of RXRs is not exactly known although methylation associated down-regulation might play a role [75]. Down-regulation of RXR, and up-regulation of the CYP3A genes was the main reason for the IPA pathway 'LPS/IL-a mediated inhibition of RXR function' to be significantly affected by the 24h subset of genes.

Pathway analysis by neither CoPub nor IPA did reveal significantly enriched pathways in the 72h subset of biomarkers. Activation of the typical genotoxicity pathways by the non-genotoxic liver toxicants is probably the reason (see results section 1). Overall pathway analysis clearly indicated that the p53 responsive element or one of the pivotal p53 downstream targets like for example CDKN1A and MDM2 might contain valuable promoters or responsive elements for the generation of reporter assays.

In conclusion, gene expression analysis with Qlucore Omics Explorer revealed a 6h, 24h, and 72h gene subset that discriminated genotoxic from non-genotoxic liver toxicants exerting their effect through cytotoxicity. The most prominent induced genes in the three subsets of genes were mainly involved in cell cycle control and downstream targets of p53. Further studies are undergoing to assess the role of the individual gene expression in luciferase based reporter assays which may provide a tool for screening genotoxicity with lower levels of false positives.

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Chapter 7

The development of RAD51C, Cystatin A, p53 and Nrf2 luciferase reporter assays in metabolically competent HepG2 cells for the assessment of mechanism-based genotoxicity and of oxidative stress in the early research phase of drug development

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Abstract

Four different mechanism-based high-throughput luciferase reporter assays were developed in human HepG2 cells, which contain phase I and II metabolic activity and a functionally active p53 protein. The promoter regions of RAD51C and Cystatin A, as well as the responsive element of the p53 protein, were selected for the generation of the genotoxicity reporter assays. Moreover, a luciferase based reporter assay was generated that measures the activation of the Nrf2 oxidative stress pathway.

Validation with respect to the ECVAM compound list [Mutat. Res 653 (2008) 99-108] resulted in an overall sensitivity of the HepG2 genotoxicity reporter assays for genotoxicity of 85% (17/20). The specificity and predictivity were high with 81% (34/42) and 82% (51/62), respectively. Various compounds had a positive score although metabolic activation was needed. The HepG2 reporter data were also compared with the available data of bacterial mutagenicity (Ames test), in vitro mammalian genotoxicity and in vivo genotoxicity for an additional set of 192 compounds. The predictivity for mutagenicity results was 74% (sensitivity, 61%, 30/49; specificity, 80%, 77/96) and for in vitro mammalian genotoxicity 59% (sensitivity, 45%, 35/78; specificity 83%, 38/46). The correlation between results from the HepG2 genotoxicity reporter assays and in vivo genotoxicity was higher with 77% (sensitivity, 74%, 28/38; specificity 81%, 26/32). Results from the Nrf2 reporter assay showed that a large number of genotoxic compounds activated the Nrf2 oxidative stress pathway.

In conclusion, four high-throughput mechanism-based reporter assays in the HepG2 cell line were developed, which can be applied for screening in the early research phase of drug development. The use of these assays may reduce the attrition rate due to genotoxicity in the developmental phase of drug development.

Introduction

Regulatory genotoxicity tests like the Ames, sister chromatid exchange (SCE), micronucleus (MN), mouse lymphoma assay (MLA) and chromosome aberration (CA) tests are time-consuming, laborious and not applicable for screening of large numbers of compounds in the early research phase of drug development. In this phase of drug development medium-throughput (~10-100 compounds/month) or high-throughput (>~100 compounds/month) screening methods are essential.

Recently, the medium throughput Vitotox™ (*Salmonella typhimurium*) and Radarscreen (yeast) assays were validated [1]. The results showed that the Vitotox™ and RadarScreen have a high predictivity for bacterial mutagenicity and in vitro mammalian genotoxicity , respectively. Especially, the number of false positive scores (due to cytotoxicity) in these in vitro assays was relatively low. The regulatory genotoxicity assays as well as the medium-throughput Vitotox™ and Radarscreen assays have the disadvantage that they are mainly non-human assays which require rat-S9 mixture for metabolic activation. Therefore these assays provide only limited amount information regarding the genotoxic mode of action in humans. Thus genotoxicity assays in human metabolically competent cell lines of human origin are demanded.

In the present study, four luciferase-based reporter assays were developed and validated in the human HepG2 cell line. The promoter regions of RAD51C and Cystatin A, and the p53 and Nrf2 responsive elements were used to control the expression of a luciferase gene, which can be quantified easily. The HepG2 cell line was chosen because it contains many functional phase I and II enzymes which are lost in most cultured cell lines [2, 3]. Moreover, the HepG2 cell line is wild-type for the tumour suppressor gene p53 [4] whereas the V79 and CHO cell lines that are commonly used in genotoxicity assays have a mutated and non-functional p53 protein (no or less DNA repair) and lack metabolic activity. The presence of (phase II) metabolism and a functionally active p53 enzyme in HepG2 cells most likely results in less falsely predicted in vivo results in comparison with results obtained with CHO and V79 cells. Other studies already showed the suitability and applicability of HepG2 cells in genotoxicity assays such as the MN and Comet assay [5-11].

DNA damage is often used as end point in genotoxicity assays. In the present study the induction of stress pathways/proteins was measured to get mechanism-based insight into the genotoxic mode(s) of action of compounds. The choice for the four pathways was based on the results of micro-array experiments with genotoxic compounds in HepG2 cells. The relevance of each of these pathways is explained in more detail in the paragraphs below.

Double strand breaks are a primary or secondary effect of exposure to many genotoxic compounds. Homologous recombination is an important mechanism to repair these

double strand breaks during the S- or G2-phase of the cell cycle [12]. In humans, several proteins have been reported to play a crucial role in this process. Important proteins are RAD51, RAD52, RAD54, replication protein A, MRE11/RAD50/NBS1, and the RAD51 paralogs RAD51B, RAD51C, RAD51D, XRCC2, and XRCC3. Following DNA damage, RAD51 accumulates at the sites where repair reactions must take place. RAD51C forms a complex that includes either XRCC2 or XRCC3 [12]. The RAD51C/XRCC2 complex plays a role in RAD51 mediated strand exchange [13] and the RAD51C/XRCC3 complex has a function in resolving Holliday junctions in the late stage of homologous recombination [14, 15]. In addition, RAD51C has been reported to have an important role in maintaining the correct number of centrosomes in mitosis [16]. Based on the microarray and literature data a luciferase-based RAD51C reporter assay was generated.

Moreover, a Cystatin A promoter luciferase-reporter assay was generated. Cystatin A is a cysteine protease inhibitor that belongs to family 1 of the cystatin superfamily. It efficiently inhibits cathepsin B with an inhibitor constant (Ki) of 8 nM [17, 18]. Unlike many other cystatins, Cystatin A does not contain a signal peptide for secretion and is consequently retained within the cell [17, 18]. Expression has been reported in polymorphonuclear granulocytes, spleen, keratinocytes, and liver [19, 20]. After treatment of keratinocytes with UVB Cystatin A caused an anti-apoptotic effect [20]. Cystatin A inhibited apoptosis caused by caspase-3 induction. Anti-apoptotic effects have also been reported in the rat hepatoma cell line H4IIE [21] where Cystatin A reduced bile salt-induced apoptosis. The induction which is seen after treatment with genotoxic compounds suggests that activation of this protein allows the cell more time for DNA repair.

The p53 tumour suppressor gene is the pivotal protein in the response to DNA damage, mitotic spindle disruption and activation of oncogenes [22]. Under non-stress situations the p53 protein is a short-lived transcription factor. Activation of the G2/M checkpoint by the ATM and ATR proteins results in phosphorylation of the checkpoint kinase Chk2. Subsequently, this kinase phosphorylates p53 which prevents the binding of p53 to mdm2 that targets p53 for ubiquitylation. Targets of the activated p53 proteins are pathways involved in cell cycle arrest, DNA repair and apoptosis. The p53 transcription factor binds to a p53 responsive element in the promoter of target genes. In the present study, a luciferase based construct containing a p53 responsive element was used to measure p53 activation.

An important stress pathway that protects the cell against genotoxic and cytotoxic compounds is the Nrf2 pathway [23-25]. Nrf2 is a basic-leucine zipper heterodimer. It has a tissue-restricted subunit, p45, complexed with a small, ubiquitous member of the Maf protein family [26, 27]. Nrf2 is a transcription factor that activates phase II detoxifying enzymes and antioxidant-stress proteins. In an environment without oxidative stress,

the Keap-1 protein binds to Nrf2 and sequesters Nrf2 in the cytoplasm. Under stress conditions, antioxidants interact with the thiol groups of Keap-1. This causes the release of Nrf2 and translocation of Nrf2 to the nucleus where it heterodimerizes with the small Maf protein. This complex binds to the antioxidant responsive elements, which can activate the expression of several genes [23]. A luciferase based reporter assay driven by the Nrf2 responsive element was used in the present study.

Recently, an ECVAM workgroup defined a list of 20 genotoxic and 42 non-genotoxic compounds [28] that can be used for the validation and/or optimization of in vitro genotoxicity assays. In the present study, this compound set was used for the evaluation of the RAD51C, Cystatin A, p53 and Nrf2 reporter assays. Moreover, an additional set of 192 compounds was used to broaden this validation study [1]. This set of compounds is described in more detail in a previous study that evaluated the Vitotox™ and RadarScreen assay (Chapter 2) [1]. The compounds of this additional set can be classified as non-genotoxins and genotoxins; the set consisted of in-house and reference compounds. The genotoxins act according to diverse mechanisms and the compound list also contains many steroidal compounds that have been reported as clastogenic or aneugenic [1, 29]. The assays performed in this study may give more insight into the genotoxic mode of action of these steroids. Results from the 192 compounds were compared with the available results on bacterial mutagenicity (Ames test), in vitro mammalian genotoxicity and in vivo genotoxicity.

Materials and methods

Preparation of compound solutions

Stock solutions of the reference and test compounds were prepared in 100% dimethyl sulfoxide (DMSO). From the stocks, √10-fold dilution series were prepared in DMSO. All compounds were of analytical grade and ordered from Sigma-Aldrich or synthesized in-house. More details on the chemical constituents of the in-house compounds are described by [1].

Cell culture

HepG2 cells were obtained from the American type culture collection (Rockville, MD, USA). HepG2 cells were cultured in Dulbecco's modified eagles medium and nutrient mixture F-12 mixed in a ratio of 1:1 (Invitrogen) with 10% defined supplemented bovine calf serum from Hyclone (UT, USA) and 1% penicillin-streptomycin (10,000 U/ml, Invitrogen). Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37 ºC and medium was refreshed every 3 or 4 days with sub-culturing.

Development of a RAD51C, and Cystatin A luciferase reporter vector

DNA was isolated from HepG2 cells with the DNeasy kit (Qiagen, Germatown, USA). PCR reactions were performed to isolate the promoter regions of RAD51C and Cystatin A. The PCR mixture contained 5 µl 10x PCR buffer, 1 µl 10 mM dNTP mixture, 1.5 µl 25 mM $MgCl_2$, 2.5 µl 5 µM forward and reverse primer, 50 ng DNA, 2.5 U Taq polymerase, and 0.25 U Pfu polymerase. Milli-Q was added to get a total volume of 50 µl. The program used was 5 min at 94ºC, followed by 30 cycles of 30 s at 94ºC, 30 s at 58ºC or 55ºC for respectively Cystatin A and RAD51C, and 1 or 2 min at 72 ºC for respectively Cystatin A and RAD51C, followed by 7 min at 72ºC.

The sequence of the human RAD51C promoter was adapted from the eukaryotic promoter database (www.epd.isb-sib.ch) [30]. The RAD51C promoter has not been analyzed in detail. However, the paralog, RAD51B (RAD51L1) has been investigated [31]. This gene is also induced after DNA damage. Analysis of this promoter showed that it contains several UV-responsive elements and - in the region 1630-1700 bp before the transcription start site - it contains two p53 binding sites separated by a spacer. This region with the p53 binding sites was essential for the UV responsiveness of the promoter. In addition this region was also essential for silencing the promoter when there was no induction by UV.

The sequence of the RAD51C promoter site starting 2100 bp downstream the transcription start site was aligned with the sequence from the RAD51B promoter. The results showed that one region in the RAD51C promoter has great similarity (>80%) with part of the RAD51B promoter. This region contained three UV responsive elements. Moreover, in the RAD51C promoter two possible p53 binding sites separated by a spacer were found at approximately 1400 bp before the transcription start site. In the present study a primer pair was used that amplified the region from 2073 bp before the transcription start until 11 bp after the transcription start site. The sequences of the primers were: Forward primer 5'-GCATAAGCATGAAATCTCCCTGAA-3'; Reverse primer 5'-CATTTCAAAGCGGAACGTC-3'.

The Cystatin A promoter was analyzed in detail in the literature [19]. The region till approximately 650 bp before the transcription start site appeared to be essential for a functional promoter. The primers used in the present study amplified the region from 700 bp before the transcription start site till the transcription start. The sequence of the primers that were used for the promoter isolation were: Forward primer 5'-CCGGTACCGGCACCAGTACTTTGCCTTCTG-3'; Reverse primer 5'-GAAGATCTTCCAGGGAAGTGTGGAGACTCG-3'.

Next the TOPO TA cloning kit (Invitrogen) was used for cloning the PCR products containing the RAD51C and Cystatin A promoters. Four microliters of PCR product were added to 1 µl salt solution and 1 µl TOPO vector. The solution was gently mixed

and incubated for 5 min at room temperature. Two microliters were used for the transformation of DH5α competent cells. Clones that appeared on the LB agar plates were checked for the insert by PCR. Clones on the plate were touched by a toothpick. The toothpick was used to inoculate 25 µl PCR reaction mixture and a tube containing 1 ml LB medium. The same PCR protocol as for the promoter isolation was used. The inoculated media from clones that were positive for the insert were added to 50 ml LB medium containing 100 µg/ml ampicillin. The following day, 3 µl bacteria suspension was used to check for the insert again. When positive for the insert, plasmid DNA was isolated and checked for the insert by PCR. The plasmid DNA was then sequenced.

The pGL4.17 vector (Promega, GenBank accession number DQ188837) was used for the generation of the reporter construct. A pGL4.17 vector needs the addition of a complete promoter including elements like for example a TATA box to drive the expression of the luciferase gene. For Cystatin A, the pGL4.17 vector and TOPO vector containing the promoter were restricted with Kpn I and Bgl II. The Kpn I and Bgl II restriction sites were introduced by the Cystatin A primers that were used. The promoter sequence of Cystatin A itself does not contain these restriction sites. Products of the restriction reaction were loaded onto an 1 % agarose gel and the restricted vector and Cystatin A promoter (700 bp) were isolated from the gel and purified with the DNA and gel band purification kit (Amersham Pharmacia Biotech). The restricted vector and promoter sequence were ligated. Then 2 µl ligation mixture was used to transform DH5α competent cells. Like after transformation with the TOPO vector, clones were checked for the insert by PCR and positive clones were used to inoculate 50 ml LB medium containing 100 µg/ml ampicillin medium. The following day plasmid DNA was isolated. For the generation of the RAD51C luciferase reporter, the pGL4.17 vector and the TOPO vector containing the promoter were restricted with Kpn I and EcoR V. These two restriction sites are present in the multiple cloning site of the TOPO vector. Moreover, the 2000 bp sequence of the promoter does not contain these restriction sites. The orientation of the insert in the TOPO vector was checked by restricting the vector with a combination of Kpn I and Bgl II. Depending on the orientation, fragments of 600, 2400, and 2800 bps (correct orientation) or 1400, 1600, and 2900 bps were formed. Plasmids with the correct orientation were selected for the experiments. The remaining part of the protocol was similar to that used for Cystatin A.

Development of a p53 and Nrf2 luciferase reporter vector

A luciferase based reporter vector containing four p53 responsive elements was generated. An oligo with the sequence 5'-GGGGTACCCC-$(AGGCATGTCT)_4$-GAAGATCTTC-3' was hybridized with the complementary sequence. Of both oligos 5 µg were added to a 1x PCR buffer without magnesium (Invitrogen) in a total volume of 50 µl in a well of a 96-well PCR plate (Applied Biosystems). The plate was put in

a GeneAmp PCR System 9700 (Applied Biosystems). The program used was 5 min at 94ºC followed by 10 min at 71ºC. The resulting DNA fragment contained four repeats of the p53 binding site 5'-AGGCATGTCT-3' and a Kpn I restriction site at the 5' end and a Bgl II restriction site at the 3' end.

For the generation of the Nrf2 reporter an oligonucleotide with the sequence 5'-GGGGTACCCCAGTCACAGTGACTCAGCAGAATCGTAAGATCTTC-3' was hybridized with its complementary sequence. The procedure was similar to that described for the generation of the p53 reporter vector.

A pGL3-promoter vector containing a firefly luciferase gene (Promega, GenBank accession number U47298) was double digested with Kpn I and Bgl II. The pGL3 vector needs only the addition of a responsive element and contains all other sequences that are necessary for a good working promoter. The same restriction reaction was performed with the p53 and Nrf2 reporter constructs. Restriction enzymes were heat inactivated by heating the samples at 70ºC for 20 min. The restriction product of the plasmid was loaded onto an 1% agarose gel and run for 1 h. Next the digested plasmid DNA was cut from the gel and purified with a DNA and Gel Band Purification kit. Thereafter, the rapid DNA ligation kit from Roche (Basel, Switzerland) was used to ligate the restricted vector and insert. One hundred ng vector (pGL3 or pGL4) and three times the equimolar amount of insert were diluted in 1x DNA dilution buffer in a volume of 10 µl. Subsequently 10 µl 2x ligase buffer and 1 µl containing 5 Weiss Units T4 DNA ligase were added. The ligation reaction was performed for 5 min at room temperature.

Then DH5α competent cells (Invitrogen) were transformed with the ligation product. Product of the ligation reaction (2µl) was added to the competent cells. Cells were incubated on ice for 30 min. The tubes were heat-pulsed in a 42 ºC water bath for 40 s and thereafter chilled on ice for 2 min. Next, 0.9 ml SOC medium (Promega) of 42 ºC was added. Cells were incubated for 1 h at 37ºC with shaking at 250 rpm. 100 µl of the transformation mixture was plated on LB agar plates containing 100 µg/ml ampicillin.

Clones that appeared on the plate were checked for an insert by PCR (Forward primer, 5'-GCAAGTGCAGGTGCCAGAAC-3'; Reverse primer, 5'-GCCTCGGCCTCTGC ATAAA-3'). Bacteria that were positive for an insert were used to inoculate 50 ml LB-medium containing 100 µg/ml ampicillin. Bacteria were grown overnight and glycerol stocks of the bacteria were prepared and plasmid DNA was isolated. Clones were again checked for an insert by PCR and a double digestion with Xho I and Bgl I. A final check for the insert was performed by sequencing.

Transfection and generation of stable single cell clones

HepG2 cells were trypsinized, counted and resuspended in culture medium to a final concentration of $3x10^4$ per well. To each well of a white 96-well culture plate (Perkin Elmer) 90 µl cell suspension was added. One 96-well plate was prepared per construct.

The plates were incubated overnight for 24 h in a humidified atmosphere at 37ºC under 5% CO_2.

Transfection mixtures were prepared by diluting Fugene-6 transfection reagent (Hoffmann-La Roche, Basel, Switzerland) with serum-free Optimem medium (Invitrogen) in a ratio of 1:16. This mixture was incubated for 5 min at room temperature. Then vectors were added resulting in a Fugene-6:plasmid DNA ratio of 6µl:1µg. In case of the p53 and Nrf2 reporter, cells were cotransfected with a pcDNA3.1+ (Invitrogen) vector. This vector harbors a neomycin resistence operon that enables the selection of stable transfectants. In case of the RAD51C_luc and Cystatin A_luc vectors a neomycin marker is already present. The Fugene-6:plasmid DNA mixture was gently shaken and incubated for 30 min at room temperature. Per well, mixture containing 100 ng plasmid DNA was added for the transfection reaction. After transfection, the plate was incubated for 24 h at 37ºC. Then standard culture medium was exchanged with culture medium containing 1% geneticin. Culture medium was refreshed every 3 or 4 days. The majority of the cells died due to the presence of geneticin, but some cell clusters survived. After two weeks the transfectants were trypsinized and homogeneously spread in an empty transparent 96-well plate. Stably transfected cells were scaled up to confluent 6-well plates. The cell pools were trypsinized and each well was resuspended in 2 ml culture medium. From this suspension eight aliquots of 50 µl were seeded in a white 96-well culture plate. The other part was transported to a Nunclon™ culture easyflask of 25 cm^2. To the white plate 40 µl medium was added and these cells were incubated for 24 h. The next day, 10 µl doxorubicin in a dose range of 10^{-5} to 10^{-8} M (√10-fold steps) or vehicle alone in DMSO was added leading to a final concentration of 0.1% DMSO. The cells were incubated for 24 h. Subsequently 50 µl LucLite reagent (Perkin Elmer) containing lysis reagent, ATP and luciferin was added and after 45 min shaking on a plate shaker the luminescence was measured for 1 s on a TopCount® NT Microplate scintillation and luminescence counter (Perkin Elmer). The 15 clones showing the highest induction were selected and scaled up to confluency in flasks of 175 cm^2. A part of the cells was stored at -80ºC with medium containing 20% DMSO. Stable single cell clones were then selected. Cells of the best 3-5 pools (>5-10 fold induction) were seeded single cell by adding 600 cells in the first well in a transparent plate and thereafter diluting them 1:2 in culture medium until the last well. Per cell pool five plates were prepared. The ultimate amount of culture medium was 200 µl in each well. The first well contained 100 µl and the last well contained 300 µl. After one and a half week single cell clones were selected by microscopic inspection and medium in these wells was refreshed every 3-4 days. After 3-4 weeks the single cell clones were transported to a transparent 96-well plate and scaled up to 6-well plates. The same protocol as for the pools was used to test the induction of luminescence after doxorubicin treatment. Then up to 10 single cell clones showing the highest induction were selected and cell stocks were frozen. Medium was

replaced by medium without geneticin and cells were cultured for two months. Every week the luciferase induction by doxorubicin was tested. Cells showing a reproducible induction over a period of two months in culture were defined as stably transfected single cell clones. Cell stocks from these cells were stored in the freezer. The stable single cell clones with the highest induction were used for compound testing. The developed cell lines were called HepG2 RAD51C_luc, Cystatin A_luc, p53_luc, and Nrf2_luc.

Evaluation of the assays with compounds

The HepG2 reporter cells were trypsinized, counted and resuspended in culture medium leading to a final concentration of $2x10^4$ cells/well (90 µl) in a white 96-well plate. The plates were incubated for 24 h in a humidified atmosphere at 37 ºC under 5% CO_2. Following the pre-incubation, seven serial dilutions (√10 steps) of the compounds or a control sample were added as 10 µl fractions to HepG2 cells leading to a final volume of 100 µl (1% DMSO). Doxorubicin and benzo[a]pyrene were used as positive controls in every assay. These compounds were added to the lanes 1 and 2 in each plate. The highest tested concentration of the compounds was 10^{-3} M. An exception was doxorubicin for which the highest tested concentration was 10^{-4} M. Compounds were tested in duplicate on two separate plates. After the addition of the compounds the plates were incubated for 48 h in a humidified atmosphere at 37 ºC under 5% CO_2. Subsequently, 50 µl LucLite reagent was added. Plates were shaken for 1 h and the luminescence was measured with the TopCount NT microplate scintillation and luminescence counter. All experiments in the present study were performed in at least two independently performed experiments. Results from one representative experiment are shown in this chapter.

The compounds that were used for the validation study are described in more detail in a Chapter 2 [1]. In vivo genotoxicity data were added for the 192 additionally tested compounds. These data was acquired from the CPDB, IARC, and ICSAS databases.

Calculations

After testing, the values of the two duplicate plates were averaged. Then the average of the 12 controls (1% DMSO) was calculated and this value was used to assess the induction factor of the compounds.

A compound was considered to be positive in the HepG2 RAD51C_luc, Cystatin A_luc and p53_luc assay when the response was induced by 1.5-fold (50% increase ≥ 5 times the standard deviation of the background). Background means here HepG2 reporter cells treated with 1% DMSO.

In total, 120 untreated controls were used to assess the threshold of 1.5-fold (derived from 10 independent experiments). In the HepG2 Nrf2_luc assay the threshold for a positive results was set at an induction factor of 2-fold. The procedure used to assess this threshold was the same as for the other reporter assays.

The highest test concentration in the assays was 1 mM. This top dose is the same as the proposed top dose for the regulatory in vitro mammalian tests (ICH guideline S2(R1), under revision). However, this revision has yet to be ratified.

Compounds that are positive in the HepG2 reporter assays can show a positive ('A') or negative results in a second test ('B'). Similarly, compounds with a negative score in the reporter assays can give a positive ('C') or negative ('D') score in a second test. The second test that is used for a comparison gives A+C positive scores and B+D negative scores. By using these values the sensitivity, specificity and predictivity/correlation were calculated: sensitivity = A/(A+C); specificity = D/(B+D); predictivity/correlation = (A+D)/(A+B+C+D).

An aspect that can cause false positive results is precipitation. Therefore, it was decided to discard measurements showing precipitation. Moreover, an Alamar Blue assay was performed to assess cytotoxicity [32]. Measurements showing more than 80% cytotoxicity were discarded. Non-transfected HepG2 cells were used for this cytotoxicity assay. It was assumed that the transfected cells showed the same level of cytotoxicity. Doxorubicin was used as reference compound. A concentration of $3.15x10^{-5}$ M doxorubicin killed almost all cells and this concentration was set at 100% toxicity.

Hierarchical clustering

Hierarchical clustering is a method that can be used to show the level of similarity between the assays or compounds. The closer the assays/compounds cluster together the higher the similarity is. In this way it is another way to present the data. reporter assay data were hierarchical clustered by using Spotfire DecisionSite version 9.1 (TIBCO). The hierarchical clustering method used was UPGMA (unweighted average). The similarity measure used was Euclidean distance.

Results

Validation of the assay procedure with reference compounds and the reproducibility of the assays

The HepG2 reporter assays were evaluated by using the ECVAM compound list and an additional set of 192 compounds. The results are described in the next two sections. The results for a representative set of compounds with diverse mechanisms, i.e. 4-nitroquinole-1-oxide (4NQO), benzo[a]pyrene (B[a]P), doxorubicin, cisplatin, 5-fluorouracil, taxol and hydrogen peroxide (H_2O_2), are shown in Figure 1. The cytotoxicity data of the compounds tested in an Alamar Blue assay are also shown in Figure 1.

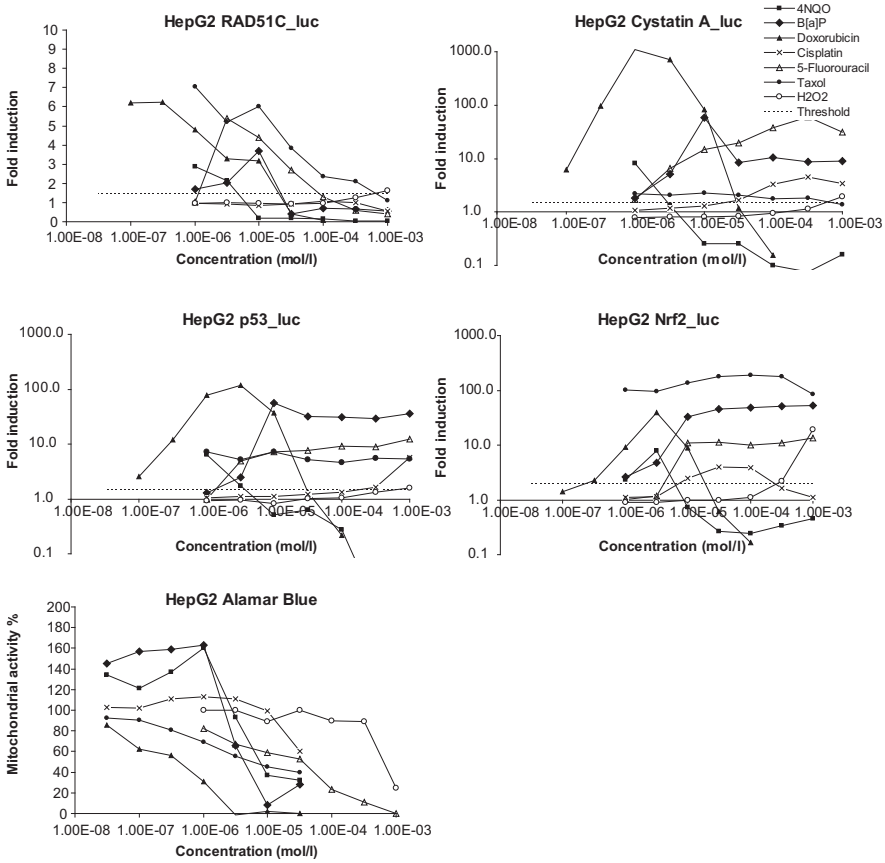

Figure 1. Concentration-dependent genotoxicity and cytotoxicity of a representative set of seven compounds in de four HepG2 reporter and Alamar Blue assays. Compounds were tested in two independently performed experiments. A representative curve is shown.

The direct-acting genotoxicant 4NQO showed induction in all four reporter assays with a maximal induction at $\leq 1.00x10^{-6}$ M or $3.16x10^{-6}$ M. After the maximum induction the luminescence drops sharply due to the cytotoxicity of 4NQO.

B[a]P which is activated by CYP1A1 showed activity in all four reporter assays. The Lowest Effective Concentration (LEC) in the HepG2 RAD51C_luc and p53_luc reporter assay was $\leq 1.00x10^{-6}$ M. Subsequently the LEC was $3.16x10^{-6}$ M in the Nrf2_luc and Cystatin A_luc reporter assay.

The cross-linking agent cisplatin showed activity in the HepG2 Cystatin A_luc, p53_luc and Nrf2_luc reporter assays with a LEC of $3.16x10^{-5}$, $3.16x10^{-4}$ M, and $1.00x10^{-5}$ M, respectively. The maximum induction in these assays was between 4-, and 5-fold.

Cisplatin showed no activity in the HepG2 RAD51C_luc assay.

The anti-metabolite 5-fluorouracil had a LEC of $3.16x10^{-6}$ M in the HepG2 RAD51C_luc, p53_luc and Nrf2_luc assay. In the HepG2 Cystatin A_luc reporter assay 5-fluorouracil showed already activity at $1.00x10^{-6}$ M. The maximum induction was respectively 5, 60, 7, and 13-fold in the HepG2 RAD51C_luc, Cystatin A_luc, p53_luc and Nrf2_luc reporter assays.

The spindle poison taxol showed already activity in all reporter assays at a concentration of $\leq 1.00x10^{-6}$ M. The maximum induction in the HepG2 RAD51C_luc, Cystatin A_luc, p53_luc, and Nrf2_luc assay was 7, 2, 55, and 102-fold, respectively.

Hydrogen peroxide causes DNA damage as a result of the formation of reactive oxygen species. Hydrogen peroxide caused an induction of the luminescence in the HepG2 RAD51C_luc, Cystatin A_luc and p53_luc reporter assays at a concentration of $1.00x10^{-3}$ M. The maximum induction in these assays was just below 2-fold. The maximum induction in the HepG2 Nrf2_luc assay was 19-fold. In this assay a significant induction was already observed at a concentration of $3.16x10^{-4}$ M.

To show the reproducibility of the reporter assays, doxorubicin and B[a]P were tested in 10 independent experiments (different compound stocks and days, same operator) (Figure 2). Doxorubicin had a LEC of $\leq 1.00x10^{-7}$ M in all 10 experiments in the HepG2 Rad51C_luc, Cystatin A_luc and p53_luc assays. In the p53_luc assay, B[a]P had an LEC of $3.16x10^{-6}$ M in 8 experiments and of $1.00x10^{-6}$ M in 2 experiments. The variation in the RAD51C_luc assay was a bit larger. B[a]P had an LEC of $1.00x10^{-6}$ M, $3.16x10^{-6}$ M, and $1.00x10^{-5}$ M in 4, 4 and 2 experiments, respectively. In the Cystatin A_luc assay the reproducibility was high with an LEC for B[a]P of $1.00x10^{-6}$ M in 6 experiments and of $3.16x10^{-6}$ M in 4 experiments. The reproducibility of the Nrf2_luc assay was also high. Doxorubicin had a LEC of $3.16x10^{-7}$ M in 7 experiments and of $1.00x10^{-6}$ M in 3 experiments. The LEC of B[a]P was $1.00x10^{-6}$ M in all 10 experiments. Overall the experiments with the 2 reference compounds gave a first indication that the reproducibility of the HepG2 reporter assays is good.

Validation of the HepG2 RAD51C_luc, Cystatin A_luc, p53_luc, and Nrf2_luc reporter assays with respect to the ECVAM compound list

The effects of the 62 ECVAM compounds were tested in the four luciferase based reporter assays. The overall results are shown in Tables 1-3. Data were compared with the genotoxic/non-genotoxic classification of the compounds. The sensitivity, specificity and predictivity of the HepG2 RAD51C_luc, Cystatin A_luc, and p53_luc reporter assays were calculated (Table 4).

The HepG2 p53_luc assay had the highest sensitivity with a score of 85%. Among the 20 genotoxic compounds cyclophosphamide, dimethylnitrosamine and chloramphenicol were not detected. Several compounds that need metabolic activation like B[a]P,

7,12-dimethylbenzanthracene, 2-acetylaminofluorene, 2,4-diaminotoluene, IQ, PhIP. HCl and aflatoxin B1 gave a positive score in the HepG2 p53_luc reporter assay. As no S9 mixture was used, this indicates that endogenous metabolism in HepG2 cells was sufficient for the metabolic activation of these compounds. On the other hand, metabolism by CYP2B6 and CYP2E1 seems to be too low to activate cyclophosphamide and dimethylnitrosamine, respectively.

Cyclophosphamide, dimethylnitrosamine, and chloramphenicol were also not detected in the RAD51C and Cystatin A_luc reporter assays. In addition PhIP.HCl, cadmium chloride, cisplatin, p-chloroaniline, and hydroquinone were not detected in the RAD51C_ luc assay indicating that double strand break repair by homologous recombination is not involved. In the Cystatin A_luc reporter assay PhIP.HCl, cadmium chloride, and p-chloroaniline came out negative. The HepG2 Rad51C_luc and Cystatin A_luc reporter assays had a sensitivity of 60% and 70%, respectively.

Among the 20 genotoxic compounds, 15 had a positive score in the Nrf2_luc reporter assay (75%) indicating that the formation of reactive intermediates might play a role in the genotoxic effects of these compounds. Cyclophosphamide, dimethylnotrosamine, PhIP.HCl, p-chloraniline, and chloramphenicol were not active in the Nrf2_luc assay.

The HepG2 RAD51C_luc, Cystatin A_luc and p53_luc reporter assays had also a high specificity for genotoxic compounds. The specificity of the RAD51C_luc, and Cystatin A_luc and p53_luc reporter assay was 93, 90 and 93%, respectively.

Of the 19 compounds that give often false positive results in vitro only propyl gallate was positive in the p53_luc assay. Although not positive curcumin showed a tendency to induction (>1.4 fold induction at $3.16x10^{-5}$ M). Propyl gallate and curcumin were positive in the RAD51C_luc assay. In the Cystatin A_luc assay tertiary-butylhydroquinone, urea, propyl gallate, and 2,4-dichlorophenol were positive.

Of the non-DNA reactive chemicals that have not been reported to give positive results in vitro (23 compounds), fluometron and phenanthrene scored positive in the p53_luc reporter assay. In the RAD51C_luc and Cystatin A_luc reporter assays only ampicillin showed a positive result.

The overall predictivity of the HepG2 RAD51C_luc, Cystatin A_luc and p53_luc reporter assays for genotoxicity was high with scores of 82, 84 and 90%, respectively while these values were 85% and 79% for the Vitotox™ and RadarScreen assays, respectively. The predictivity for genotoxicity was 82% (sensitivity 85%; specificity 81%) when the three reporter assays were combined. The percentage of Nrf2 positive compounds in the group of the non-genotoxic compounds was 31% (13/42). This percentage is much lower than the 75% measured for the group of genotoxic compounds.

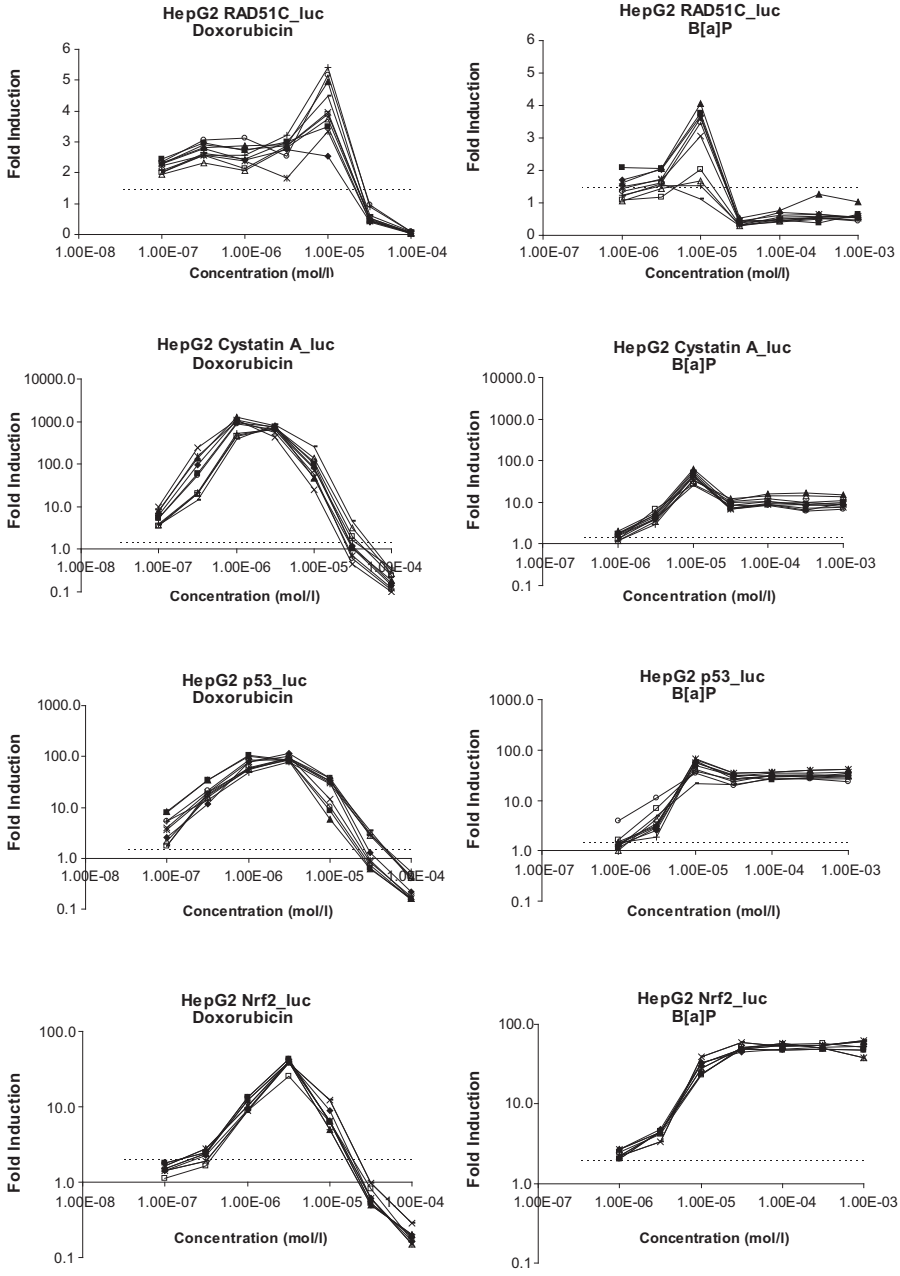

Figure 2. The reproducibility of the concentration dependent effects of doxorubicin and benzo[a]pyrene (B[a]P) in the four HepG2 reporter assays in ten independently performed experiments. The dashed line represents the genotoxicity threshold.

Table 1. Results from the RAD51C, Cystatin A, p53 and Nrf2 luciferase reporter assays regarding the ECVAM compounds that were defined as in vivo genotoxins which should be detected as positive in in vitro mammalian cell genotoxicity tests. The results from the previously validated Vitotox™ and RadarScreen assays are also shown [1]. The false negative results are indicated in bold.

Chemical	CAS number	Further information	Vitotox	Radar Screen	RAD51C luc	LEC (mol/l)	Cystatin A luc	LEC (mol/l)	p53 luc	LEC (mol/l)	Nrf2 luc	LEC (mol/l)
I. Ames-positive in vivo genotoxins												
(i) O^6 and N^7 alkylators												
Cyclophosphamide	6055-19-2	Requires metabolic activation (CYP2B6)	**N**	Y (S9)[a]	**N**		**N**		**N**		**N**	
ENU	759-73-9	Strong gene mutagen (O^6 alkylation)	Y	Y	Y	$1.00x10^{-3}$	Y	$1.00x10^{-3}$	Y	$3.16x10^{-4}$	Y	$1.00x10^{-3}$
MMS	66-27-3	Strong clastogen (N^7 alkylation)	Y	Y	Y	$\leq 1.00x10^{-6}$	Y	$\leq 1.00x10^{-6}$	Y	$\leq 1.00x10^{-6}$	Y	$1.00x10^{-4}$
(ii) Polycyclic aromatic hydrocarbons												
Benzo[a]pyrene	50-32-8	Requires metabolic activation (CYP 1A1; 1B1, epoxide hydrolase); forms bulky adducts	Y(S9)	Y(S9)[a]	Y	$1.00x10^{-5}$	Y	$3.16x10^{-5}$	Y	$1.00x10^{-5}$	Y	$\leq 1.00x10^{-6}$
7,12-Dimethylbenzanthracene	57-97-6	Requires metabolic activation (CYP1B1); forms bulky adducts	Y	Y	Y	$1.00x10^{-5}$	Y	$1.00x10^{-5}$	Y	$1.00x10^{-5}$	Y	$1.00x10^{-5}$
(iii) Aromatic amines												
Dimethylnitrosamine	62-75-9	Alkylating agent after activation by CYP2E1(which is not highly expressed in rat liver S9): produces O^6- and N^7-methyl guanine adducts	**N**	Y(S9) [a]	**N**		**N**		**N**		**N**	
2-Acetylaminofluorene	53-96-3	Hydroxylated by CYP1A2 and then acetylated. Forms C8 adduct on guanine	Y(S9)	Y(S9)	Y	$1.00x10^{-4}$	Y	$1.00x10^{-4}$	Y	$3.16x10^{-5}$	Y	$1.00x10^{-3}$
2,4-diaminotoluene	95-80-7	Aromatic amine, requires metabolic activation	Y(S9)	Y(S9)	Y	$3.16x10^{-4}$	Y	$3.16x10^{-4}$	Y	$3.16x10^{-4}$	**N**	
IQ (2-amino-3methyl imidazo[4,5-*f*]quinoline)	76180-96-6	Heterocyclic amine with potent genotoxicity, requires metabolic activation	Y(S9)	Y(S9)	Y	$3.16x10^{-5}$	Y	$3.16x10^{-4}$	Y	$1.00x10^{-4}$	Y	$1.00x10^{-3}$
PhIP.HCl (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine		Heterocyclic amine with potent genotoxicity, requires metabolic activation	Y	**N**	**N**		**N**		Y	$1.00x10^{-4}$	**N**	
(iv) Others												
Aflatoxin B1	1162-65-8	Activated by CYP3A4, which is not highly expressed in rats compared with humans. Forms various adducts	Y	Y(S9)	Y	$1.00x10^{-5}$	Y	$3.16x10^{-6}$	Y	$3.16x10^{-6}$	Y	$3.16x10^{-5}$
Cadmium chloride	10108-64-2	Inorganic carcinogen	**N**	Y	**N**		**N**		Y	$1.00x10^{-5}$	Y	$3.16x10^{-6}$
Cisplatin	15663-27-1	Cross-linking agent	Y(S9)	Y	**N**		Y	$3.16x10^{-5}$	Y	$3.16x10^{-4}$	Y	$3.16x10^{-5}$
p-Chloroaniline	106-47-8	No adducts	Y(S9)[a]	Y(S9)	**N**		**N**		Y	$1.00x10^{-3}$	**N**	
II. In vivo genotoxins negative or equivocal in Ames												
Etoposide	33419-42-0	Topoisomerase inhibitor	Y(S9)	Y	Y	$1.00x10^{-3}$	Y	$1.00x10^{-3}$	Y	$1.00x10^{-3}$	Y	$1.00x10^{-3}$
Hydroquinone	123-31-9	MOA: aneugen	Y(S9)	**N**	**N**		Y	$1.00x10^{-5}$	Y	$1.00x10^{-4}$	Y	$1.00x10^{-5}$
Azidothymidine	30516-87-1	MOA: nucleoside analogue	Y	**N**	Y	$3.16x10^{-4}$	Y	$3.16x10^{-5}$	Y	$3.16x10^{-4}$	Y	$1.00x10^{-4}$
Sodium arsenite	7784-46-5	Inorganic carcinogen MOA: oxidant? repair inhibitor?	**N**	**N**	Y	$1.00x10^{-4}$	Y	$1.00x10^{-4}$	Y	$3.16x10^{-4}$	Y	$1.00x10^{-4}$
Taxol	33069-62-4	MOA: aneugen	**N**	**N**	Y	$\leq 1.00x10^{-6}$	Y	$\leq 1.00x10^{-6}$	Y	$\leq 1.00x10^{-6}$	Y	$\leq 1.00x10^{-6}$
Chloramphenicol	56-75-7	MOA: clastogen that binds to DNA	**N**	**N**	**N**		**N**		**N**		Y	$1.00x10^{-3}$

[a] compounds that are activated by S9 mixture but already show some activity without the addition of S9 mixture

Table 2. Results from the RAD51C, Cystatin A, p53 and Nrf2 luciferase reporter assays regarding the ECVAM compounds that were defined as non-DNA-reactive chemicals (including non-genotoxic carcinogens), metabolic poisons and others that should give negative results in in vitro genotoxicity tests but have been reported to induce chromosomal aberrations or tk mutations in mouse lymphoma cells, often at high concentrations or at high levels of cytotoxicity. The results from the previously validated Vitotox™ and RadarScreen assays are also shown [1]. The false positive results are indicated in bold.

Chemical	CAS number	Vitotox	Radar Screen	RAD51C luc	LEC (mol/l)	Cystatin A luc	LEC (mol/l)	p53 luc	LEC (mol/l)	Nrf2 luc	LEC (mol/l)
I. Non-carcinogens that are negative or equivocal for genotoxicity in vivo											
D,L-Menthol	15356-70-4	N	N	N		N		N		**Y**	$3.16x10^{-4}$
Phthalic anhydride	85-44-9	N	N	N		N		N		N	
Tertiary-butylhydroquinone	1948-33-0	**Y(S9)**	**Y(S9)**[a]	N		**Y**	$1.00x10^{-5}$	N		**Y**	$1.00x10^{-5}$
o-Anthranilic acid	118-92-3	N	N	N		N		N		N	
1,3-Dihydroxybenzene (resorcinol)	108-46-3	N	N	N		N		N		N	
2-Ethyl-1,3-hexanediol	94-96-2	N	N	N		N		N		N	
Sulfisoxazole	127-69-5	N	N	N		N		N		N	
II. Non-carcinogens with no in vivo genotoxicity data											
Ethionamide	536-33-4	N	N	N		N		N		N	
Curcumin	458-37-7	N	Y	**Y**	$1.00x10^{-5}$	N		N		**Y**	$3.16x10^{-5}$
Benzyl alcohol	100-51-6	N	N	N		N		N		**Y**	$1.00x10^{-4}$
Urea	57-13-6	N	N	N		**Y**	$1.00x10^{-3}$	N		**Y**	$3.16x10^{-5}$
III. Non-genotoxic carcinogens or carcinogenic by irrelevant (for humans) mechanism											
Sodium saccharin	128-44-9	N	N	N		N		N		**Y**	$1.00x10^{-4}$
IV. Supplementary list (prediction of in vitro genotoxicity results less clear)											
Propyl gallate	121-79-9	N	N	**Y**	$1.00x10^{-5}$	**Y**	$1.00x10^{-4}$	**Y**	$3.16x10^{-5}$	**Y**	$1.00x10^{-5}$
p-Nitrophenol	100-02-7	N	N	N		N		N		N	
Sodium xylene sulfonate	1300-72-7	N	Y	N		N		N		N	
Ethyl acrylate	140-88-5	N	N	N		N		N		N	
Eugenol	97-53-0	N	N	N		N		N		N	
Isobutyraldehyde	78-84-2	N	N	N		N		N		N	
2,4-Dichlorophenol	120-83-2	**Y**	**Y**	N		**Y**	$3.16x10^{-5}$	N		**Y**	$1.00x10^{-5}$

[a] compounds that are activated by S9 mixture but already show some activity without the addition of S9 mixture

Table 3. Results from the RAD51C, Cystatin A, p53 and Nrf2 luciferase reporter assays regarding the ECVAM compounds that were defined as non-DNA-reactive chemicals (including non-genotoxic carcinogens). The results from the previously validated Vitotox™ and RadarScreen assays are also shown [1]. The false positive results are indicated in bold.

Chemical	CAS number	Vitotox	Radar Screen	RAD51C luc	LEC (mol/l)	Cystatin A luc	LEC (mol/l)	p53 luc	LEC (mol/l)	Nrf2 luc	LEC (mol/l)
I. Non-carcinogens with negative in vivo genotoxicity data											
Ampicillin trihydrate	7177-48-2	N	**Y(S9)**	**Y**	$1.00x10^{-6}$	N		N		N	
D-mannitol	69-65-8	N	N	N		N		N		N	
II. Non-carcinogens with no in vivo genotoxicity data											
Phenformin HCl	834-28-6	N	N	N		N		N		N	
n-Butyl chloride	109-69-3	N	N	N		N		N		N	
(2-chloroethyl)trimethyl-ammonium chloride	999-81-5	N	N	N		N		N		N	
Cyclohexanone	108-94-1	N	N	N		N		N		N	
N,N-dicyclohexyl thiourea	1212-29-9	N	N	N		N		N		N	
Trisodium EDTA trihydrate	150-38-9	N	N	N		N		N		N	
Ephidrine sulphate	134-72-5	N	N	N		N		N		N	
Erythromycin stearate	643-22-1	N	N	N		N		N		**Y**	$1.00x10^{-3}$
Fluometron	2164-17-2	N	N	N		N		**Y**	$1.00x10^{-3}$	**Y**	$1.00x10^{-3}$
Phenanthrene	85-01-8	**Y(S9)** [a]	N	N		N		**Y**	$1.00x10^{-3}$	**Y**	$1.00x10^{-3}$
III. Non-genotoxic carcinogens											
D-Limonene	5989-27-5	N	N	N		N		N		**Y**	$1.00x10^{-3}$
Di-(2-ethylhexyl)phthalate	117-81-7	N	N	N		N		N		N	
Amitrole	61-82-5	N	N	N		N		N		**Y**	$1.00x10^{-4}$
Tert-butyl alcohol	75-65-0	N	N	N		N		N		N	
Diethanolamine	111-42-2	N	N	N		N		N		N	
Melamine	108-78-1	N	N	N		N		N		N	
Methyl carbamate	598-55-0	N	N	N		N		N		N	
Progesterone	57-83-0	N	**Y**	N		N		N		N	
Pyridine	110-86-1	N	N	N		N		N		N	
Tris(2-ethylhexyl)phosphate	78-42-2	N	N	N		N		N		N	
Hexachloroethane	67-72-1	N	**Y**	N		N		N		N	

[a] compounds that are activated by S9 mixture but already show some activity without the addition of S9 mixture

Table 4. The sensitivity (Sens), specificity (Spec) and predictivity (Pred) of the Vitotox™, RadarScreen, and HepG2 reporter assays with respect to the ECVAM compound list.

	Vitotox	Radar Screen	HepG2 genotox reporters				Nrf2
			RAD51C	Cystatin A	p53	Total	
Sens (%)	70 (14/20)	70 (14/20)	60 (12/20)	70 (14/20)	85 (17/20)	85 (17/20)	75 (15/20)
Spec (%)	93 (39/42)	83 (35/42)	93 (39/42)	90 (38/42)	93 (39/42)	81 (34/42)	69 (29/42)
Pred (%)	85 (53/62)	79 (49/62)	82 (51/62)	84 (52/62)	90 (56/62)	82 (51/62)	71 (44/62)

Evaluation of the HepG2 reporter assays with respect to the additional set of 192 compounds

Besides with the compounds from the ECVAM list, the assays were further validated with an additional set of 192 compounds. The data set was not complete for all of these chemicals. Bacterial mutagenicity (Ames test) data were available for 145 compounds, in vitro mammalian genotoxicity data for 124 compounds, and in vivo genotoxicity data for 70 compounds. Moreover, for all compounds Vitotox™ and RadarScreen data are available. The results from the HepG2 RAD51C_luc, Cystatin A_luc, p53_luc and Nrf2_luc reporter assays and additional genotoxicity data available for these compounds are shown in Tables 5 and 6. The number of positive scores in the different compound groups are shown in Table 7.

Validation of the HepG2 RAD51C_luc reporter assay

Of the 192 tested compounds, 22 (11%) had a positive result in the HepG2 RAD51C_luc assay. Of these, 19 compounds were genotoxic in the regulatory genotoxicity tests. Similar to what was observed with the ECVAM compound list, the indicates the high specificity of this assay.

Sulfamoxole had a positive result in the RAD51C_luc reporter assay with a LEC of $3.16x10^{-5}$ M, but, no bacterial mutagenicity or mammalian genotoxicity data were available for this compound. The Vitotox™ and RadarScreen assays showed a negative result for this compound. As also the p53_luc and Cystatin A_luc reporter assay in HepG2 cells came out negative for this compound, the positive result in the RAD51C_luc assay was quite surprising. The Nrf2_luc assay showed a positive response, but this effect was only seen at the much higher concentration of $1.00x10^{-3}$ M.

Of the compounds with equivocal genotoxicity data in the regulatory assays, Org 9217 and Org 9935 showed a positive response in the RAD51C_luc reporter assay. These compounds showed also a positive result in the p53_luc reporter assay.

Table 5. Results from the additional 192 compounds in the HepG2 reporter assays and available data for the Vitotox™, RadarScreen, Ames assay and in vitro/in vivo mammalian genotoxicity tests. In this table the genotoxic compounds are shown.

Chemical	CAS number	Further information	Vitotox	Radar Screen	Ames	Mammalian gen. in vitro	Mammalian gen. in vivo	RAD51C luc	LEC (mol/l)	Cystatin A luc	LEC (mol/l)	p53 luc	LEC (mol/l)	Nrf2 luc	LEC (mol/l)
(i) Ames positive compounds															
1-Nitropyrene	5522-43-0		Y	Y	Y	Y	Y	Y	$3.16x10^{-5}$	Y	$1.00x10^{-5}$	Y	$1.00x10^{-5}$	Y	$3.16x10^{-4}$
2,4-Dinitrophenol	51-28-5		Y	Y	Y	Y	ND	N		N		Y	$3.16x10^{-4}$	Y	$1.00x10^{-4}$
2,7-Dinitrofluorene	5405-53-8		Y	Y	Y	Y	Y	Y	$1.00x10^{-4}$	Y	$1.00x10^{-4}$	N		N	
2-amino-3-methyl-3H-imidazo-[4,5-f]-quinoline	76180-96-6		Y(S9)[a]	N	Y	Y	Y	N		Y	$1.00x10^{-3}$	Y	$1.00x10^{-3}$	Y	$1.00x10^{-4}$
2-Hydroxy-Estradiol	362-05-0	Catechol estrogen	Y(S9)	Y	Y	Y/N	Y	N		N		Y	$1.00x10^{-4}$	Y	$1.00x10^{-5}$
2-Hydroxy-Estrone	362-06-1	Catechol estrogen	Y(S9)	Y	Y	Y/N	Y	N		Y	$1.00x10^{-5}$	Y	$1.00x10^{-5}$	Y	$1.00x10^{-5}$
2-Methoxy-Estradiol	362-07-2	Catechol estrogen	Y(S9)	Y	Y	Y	Y	N		N		Y	$1.00x10^{-3}$	Y	$1.00x10^{-5}$
2-Methoxy-Estrone	362-08-3	Catechol estrogen	N	Y	Y	Y/N	Y	N		Y	$1.00x10^{-4}$	Y	$3.16x10^{-4}$	Y	$1.00x10^{-5}$
3-Methylcholanthrene	56-49-5		Y	Y	Y	Y/N	Y	Y	$3.16x10^{-5}$	Y	$3.16x10^{-5}$	Y	$3.16x10^{-5}$	Y	$1.00x10^{-4}$
4-Hydroxy-Estradiol	5976-61-4	Catechol estrogen	N	Y	Y	Y/N	Y	N		Y	$3.16x10^{-4}$	Y	$3.16x10^{-4}$	Y	$3.16x10^{-5}$
4-Hydroxy-Estrone	3131-23-5	Catechol estrogen	Y(S9)	Y	Y	Y/N	Y	N		Y	$1.00x10^{-4}$	Y	$1.00x10^{-4}$	Y	$1.00x10^{-4}$
4NQO	56-57-5		Y	Y	Y	Y	Y	Y	$\leq 1.00x10^{-6}$	Y	$\leq 1.00x10^{-6}$	Y	$\leq 1.00x10^{-6}$	Y	$\leq 1.00x10^{-6}$
Dacarbazine	224-396-1		Y	Y	Y	Y/N	ND	N		Y	$3.16x10^{-5}$	N		Y	$1.00x10^{-3}$
Dantrolene	14663-23-1		Y	N	Y	ND	ND	N		N		Y	$3.16x10^{-4}$	Y	$3.16x10^{-6}$
Doxorubicin	25316-40-9	TOPO	Y	Y	Y	Y	Y	Y	$1.00x10^{-6}$	Y	$\leq 1.00x10^{-7}$	Y	$\leq 1.00x10^{-7}$	Y	$3.16x10^{-7}$
Ellipticin	519-23-3	TOPO	Y	Y(S9)[a]	Y	ND	Y	Y	$1.00x10^{-5}$	Y	$1.00x10^{-5}$	Y	$3.16x10^{-6}$	Y	$3.16x10^{-4}$
Hydralazine	304-20-1	Intercalating	N	N	Y	N	Y	N		Y	$1.00x10^{-4}$	Y	$1.00x10^{-3}$	N	
Hydrogen peroxide	7722-84-1	ROS	Y	Y	Y	Y	ND	Y	$1.00x10^{-3}$	Y	$1.00x10^{-3}$	Y	$1.00x10^{-3}$	Y	$3.16x10^{-4}$
β-Naphthoflavone	6051-87-2		Y	N	Y	N	ND	Y	$1.00x10^{-6}$	Y	$\leq 1.00x10^{-6}$	Y	$\leq 1.00x10^{-6}$	Y	$1.00x10^{-5}$
Melphalan	148-82-3		N	N	Y	Y	ND	Y	$1.00x10^{-4}$	Y	$3.16x10^{-6}$	Y	$1.00x10^{-5}$	Y	$3.16x10^{-4}$
Methampyrone	68-89-3	TOPO, intercalating	Y	N	Y	ND	Y	Y	$1.00x10^{-3}$	Y	$3.16x10^{-4}$	Y	$1.00x10^{-3}$	Y	$3.16x10^{-4}$
Nitrofurantoin	67-20-9	ROS	Y(S9)	Y	Y	Y/N	Y	N		N		Y		Y	$3.16x10^{-4}$
Org 20494			Y	Y	Y	ND	N	N		Y	$3.16x10^{-4}$	Y	$3.16x10^{-4}$	Y	$1.00x10^{-4}$
Org 2249			Y(S9)	N	Y	ND	ND	N		N		N		N	
Org 2408	85750-29-4		Y(S9)	Y(S9)	Y	ND	ND	N		N		N		Y	$3.16x10^{-6}$
Org 2508			Y	N	Y	ND	ND	N		N		N		N	
Org 32018			Y(S9)	N	Y	ND	ND	N		N		N		N	
Org 3240			Y(S9)	Y(S9)	Y	Y	N	N		N		N		Y	$1.00x10^{-3}$
Org 4122	67363-15-9		Y	N	Y	ND	N	Y	$3.16x10^{-5}$	Y	$3.16x10^{-5}$	Y	$1.00x10^{-5}$	Y	$1.00x10^{-4}$
Org 42671			Y	Y(S9)[a]	Y	ND	ND	N		N		N		Y	$3.16x10^{-5}$
Org 4330			Y(S9)	N	Y	ND	ND	N		N		N		N	
Org 5694			Y(S9)[a]	Y	Y	ND	ND	N		N		N		Y	$1.00x10^{-3}$
Org 5695			Y(S9)[a]	Y	Y	ND	ND	Y	$3.16x10^{-4}$	Y	$3.16x10^{-4}$	N		Y	$3.16x10^{-4}$
Org 5697			N	N	Y	ND	ND	N		N		N		Y	$3.16x10^{-4}$
Org 5710			Y	N	Y	ND	ND	N		N		N		Y	$3.16x10^{-4}$
Org 5741			Y	N	Y	ND	ND	N		N		N		N	

Table 5. Continued

Chemical	CAS number	Further information	Vitotox	Radar Screen	Ames	Mammalian gen. in vitro	Mammalian gen. in vivo	RAD51C luc	LEC (mol/l)	Cystatin A luc	LEC (mol/l)	p53 luc	LEC (mol/l)	Nrf2 luc	LEC (mol/l)
Org 5784			Y(S9)[a]	N	Y	ND	ND	N		N		N		Y	$1.00x10^{-3}$
Org 5796			Y(S9)[a]	N	Y	ND	ND	N		N		N		Y	$1.00x10^{-5}$
Org 5867			Y(S9)	N	Y	ND	ND	N		N		N		N	
Org 5907			Y	N	Y	ND	ND	N		N		N		N	
Org 7797			Y(S9)	Y	Y	N	N	N		N		N		N	
Org 9063			Y(S9)	N	Y	N	ND	N		N		N		N	
Org 9150			Y	Y	Y	ND	ND	Y	$3.16x10^{-5}$	Y	$3.16x10^{-5}$	Y	$3.16x10^{-5}$	Y	$1.00x10^{-4}$
Org 9250			Y	Y (S9)[a]	Y	ND	ND	Y	$1.00x10^{-5}$	Y	$3.16x10^{-5}$	Y	$1.00x10^{-5}$	Y	$3.16x10^{-5}$
Org 9252			Y(S9)	N	Y	ND	ND	N		Y	$1.00x10^{-5}$	Y	$1.00x10^{-3}$	Y	$3.16x10^{-5}$
Salicylamide	65-45-2		Y(S9)	N	Y	Y	N	Y	$3.16x10^{-4}$	N		Y	$1.00x10^{-3}$	N	
Tacrine	1684-40-8		N	Y	Y	Y/N	N	N		N		Y	$1.00x10^{-4}$	Y	$3.16x10^{-6}$
UK-57400	99470-74-3		Y	N	Y	ND	ND	N		N		N		Y	$3.16x10^{-5}$
Uramustine	66-75-1		N	Y	Y	ND	ND	N		N		Y	$1.00x10^{-3}$	Y	$3.16x10^{-6}$
(ii) Ames equivocal and in vitro or in vivo mammalian genotoxic															
5-Fluorouracil	51-21-8	Anti-metabolite	N	Y	Y/N	Y	Y	Y	$3.16x10^{-6}$	Y	$\leq 1.00x10^{-6}$	Y	$3.16x10^{-6}$	Y	$1.00x10^{-5}$
(iii) Ames negative or ND and in vitro or in vivo mammalian genotoxic															
16α-Hydroxy-Estrone	18186-49-7	Estrogen	N	Y(S9)[a]	N	Y/N	ND	N		N		Y	$1.00x10^{-3}$	Y	$3.16x10^{-5}$
17α-Methyltestosterone	58-18-4	Androgen	N	Y	ND ICSAS	Y/N	ND	N		N		N		Y	$3.16x10^{-5}$
Acetaminophen	103-90-2		N	N	N	Y/N	Y/N	Y	$1.00x10^{-3}$	Y	$1.00x10^{-3}$	Y	$1.00x10^{-3}$	N	
Allylestrenol	432-60-0	Progestin	N	Y	ND	Y/N	N	N		N		Y	$1.00x10^{-3}$	Y	$3.16x10^{-5}$
Bromobenzene	108-86-1	ROS	N	N	N	Y/N	N	N		N		N		Y	$1.00x10^{-3}$
Canrenoate K+	2181-04-6	Progestin	N	N	N	Y/N	N	N		N		N		Y	$3.16x10^{-5}$
Carbon tetrachloride (CCl_4)	56-23-5	ROS	N	N	N	Y/N	Y	N		N		N		N	
Chlormadinone	302-22-7	Progestin	N	Y	N	Y/N	Y (ROS)	N		N		N		Y	$3.16x10^{-5}$
Chlormadinone acetate	302-22-7	Progestin	N	Y	ND	Y/N	Y	N		N		N		N	
Chlorpromazine	69-09-0	N-dialkyl	Y(S9)	Y	N	Y/N	ND	N		N		N		N	
Chlorprothixene citrate	113-59-7	N-dialkyl	N	Y	N	Y	ND	N		N		N		N	
Clomiphene	50-41-9	SERM, N-dialkyl	N	Y	ND	Y	Y	N		N		N		N	
Colchicine	64-86-8	Spindle poison	N	N	N	Y/N	Y	N		Y	$\leq 1.00x10^{-6}$	Y	$\leq 1.00x10^{-6}$	Y	$\leq 1.00x10^{-6}$
Cyproterone acetate	427-51-0	Progestin	N	Y	N	Y/N	N	N		N		N		N	
Cytarabine	147-94-4	Anti-metabolite	N	N	N	Y	Y	Y	$\leq 1.00x10^{-6}$	Y	$\leq 1.00x10^{-6}$	Y	$\leq 1.00x10^{-6}$	Y	$3.16x10^{-6}$
Dexamethasone	50-02-2	Glucocorticoid	N	N	N	Y	Y	N		N		N		N	
Diclofenac	15307-79-6		N	Y	N	Y/N	Y	N		Y	$1.00x10^{-4}$	Y	$1.00x10^{-4}$	Y	$3.16x10^{-4}$
Diethylstilbestrol		Estrogen	N	Y	N	Y	Y	N		N		Y	$3.16x10^{-5}$	Y	$\leq 1.00x10^{-6}$
Diethylthio carbamic acid	148-18-5		N	N	N	Y/N	ND	N		N		N		Y	$3.16x10^{-4}$
Dihydroergotamine	511-12-6		N	Y	N	Y	ND	N		N		N		N	
Drospirenone	67392-87-4	Estrogen	N	Y	N	Y/N	ND	N		N		N		N	
Dydrogesterone	152-62-5	Progestin	N	Y	ND	Y/N	N	N		N		N		N	
Equilin	474-86-2	Estrogen	Y(S9)	Y	ND	Y/N	ND	N		N		Y	$3.16x10^{-5}$	Y	$3.16x10^{-5}$

Table 5. Continued

Chemical	CAS number	Further information	Vitotox	Radar Screen	Ames	Mammmalian gen. in vitro	Mammmalian gen. in vivo	RAD51C luc	LEC (mol/l)	Cystatin A luc	LEC (mol/l)	p53 luc	LEC (mol/l)	Nrf2 luc	LEC (mol/l)
Equilin-7a-methyl		Estrogen	Y(S9)	Y	ND	Y	ND	N		N		Y	$3.16x10^{-5}$	Y	$3.16x10^{-5}$
Estradiol-17α	57-91-0	Estrogen	N	Y	ND	Y/N	Y	N		N		Y	$1.00x10^{-3}$	Y	$1.00x10^{-6}$
Estradiol-17β	50-28-2	Estrogen	N	Y	N	Y/N	Y	N		N		Y	$3.16x10^{-4}$	Y	$1.00x10^{-4}$
Estriol	50-27-1	Estrogen	N	Y	ND	Y/N	ND	N		N		N		N	
Estrone (E1)	53-16-7	Estrogen	N	Y	ND	Y/N	ND	N		N		N		N	
Ethinylestradiol-17β	57-63-6	Estrogen	N	Y	N	Y/N	Y/N	N		Y	$1.00x10^{-3}$	Y	$1.00x10^{-5}$	Y	$1.00x10^{-5}$
Hexachlorobutadiene	87-68-3		N	N	N	N	Y	N		Y	$1.00x10^{-5}$	Y	$1.00x10^{-3}$	Y	$\leq 1.00x10^{-6}$
Hydrochlorothiazide	58-93-5		N	N	N	Y/N	ND	N		N		N		N	
Hydroxychloroquine sulfate	58-93-5		N	Y	ND	Y	ND	N		N		N		Y	$3.16x10^{-4}$
ICI 164.384	98007-99-9	Anti-estrogen	N	Y(S9)	ND	Y	N	N		N		N		N	
Imipramine HCl	113-52-0	N-Dialkyl	N	N	N	Y/N	Y	N		N		N		Y	$3.16x10^{-5}$
Levonorgestrel	797-63-7	Progestin	N	Y	N	Y/N	ND	N		N		N		N	
Medroxyprogesterone	520-85-4	Progestin	N	Y	N	Y/N	N	N		N		N		N	
Megestrol acetate	595-33-5	Estrogen	N	Y	ND	Y/N	Y/N	N		N		N		N	
Ment-bucyclate		Androgen	N	N	N	Y	ND	N		N		N		N	
Mestranol	72-33-3	Estrogen	N	NA	N	Y/N	Y	N		Y	$1.00x10^{-4}$	Y	$1.00x10^{-4}$	Y	$3.16x10^{-5}$
Methotrexate	59-05-2	Anti-metabolite	N	Y	N	Y	Y	Y	$3.16x10^{-6}$	Y	$\leq 1.00x10^{-6}$	Y	$\leq 1.00x10^{-6}$	Y	$\leq 1.00x10^{-6}$
Moxestrol	34816-55-2	Estrogen	N	Y	ND	Y	ND	N		N		N		N	
Norethisterone	68-22-4	Progestin	N	Y	N	Y/N	Y/N	N		N		N		Y	$3.16x10^{-4}$
Norethynodrel	68-23-5	Progestin	N	Y	N	Y/N	N	N		N		N		Y	$1.00x10^{-3}$
Noscapine HCl	912-60-7	Aneugen	N	N	N	Y	Y	N		Y	$1.00x10^{-4}$	Y	$3.16x10^{-5}$	Y	$1.00x10^{-5}$
Org 10325			N	N	N	Y	ND	N		N		N		Y	$1.00x10^{-4}$
Org 30029			Y	Y	N	Y	N	N		N		N		N	
Org 30251			Y(S9)	Y	N	Y	ND	N		N		N		N	
Org 39735	319203-75-3	Androgen	N	Y	N	Y	N	N		N		N		N	
Org 4433	68598-93-6	Progestin	N	Y	N	Y	ND	N		N		N		Y	$3.16x10^{-5}$
Oxymethalone	434-07-1	Androgen	N	Y	N	Y/N	ND	N		N		N		Y	$1.00x10^{-5}$
Rifampicin	13292-46-1		N	Y	N	Y	ND	N		N		N		N	
Rotenone	83-79-4		N	N	N	Y/N	ND	N		N		N		Y	$1.00x10^{-3}$
Stanozolol	10418-03-8	Androgen	N	Y	ND	Y/N	ND	N		N		N		N	
Sulfinpyrazone	57-96-5		N	Y	N	Y	ND	N		N		N		N	
Tamoxifen	10540-29-1	anti estrogen	N	N	N	Y	Y	N		N		N		N	
Testosterone	58-22-0	Androgen	N	Y	ND	Y/N	N	N		N		N		N	
Tolcapone	134308-13-7	Catechol	N	Y	N	Y/N	ND	N		N		N		N	
Trenbolone	10161-33-8	Androgen	N	Y	N	Y/N	ND	N		Y	$3.16x10^{-4}$	N		Y	$1.00x10^{-5}$

[a] compounds that are activated by S9 mixture but already show some activity without the addition of S9 mixture

Table 6. Results from the additional 192 compounds in the HepG2 reporter assays and available data for the Vitotox™, RadarScreen, Ames assay and in vitro/in vivo mammalian genotoxicity tests. The non-genotoxic compounds, compounds from which no genotoxicity data are available, and compounds that are equivocal for genotoxicity are summarized in this table.

Chemical	CAS number	Further information	Vitotox	Radar Screen	Ames	Mammalian gen. in vitro	Mammalian gen. in vivo	RAD51C luc	LEC (mol/l)	Cystatin A luc	LEC (mol/l)	p53 luc	LEC (mol/l)	Nrf2 luc	LEC (mol/l)
II. Non-genotoxic or no genotoxicity reported															
2,5-Hexanedione	110-13-4		N	N	ND	ND	ND	N		N		N		N	
7α-Methylnorthisterone		Androgen	N	Y	ND	ND	ND	N		N		N		Y	$3.16x10^{-5}$
Acetylsalicylic acid	50-78-2		N	N	N	N	N	N		N		N		N	
Aminophylline	317-34-0		N	N	ND	N	ND	N		N		N		Y	$3.16x10^{-4}$
Amiodarone	19774-82-4	N-Dialkyl	N	N	N	N	ND	N		N		N		N	
Antazoline mesylate	2508-72-7		N	N	ND	ND	ND	N		N		N		Y	$3.16x10^{-4}$
Atamestane	96301-34-7	Anti-estrogen	N	Y	N	N	ND	N		N		N		N	
Atropine Sulfate	5908-99-6		N	N	N	ND	ND	N		N		N		Y	$3.16x10^{-4}$
Bishydroxycoumarin	66-76-2		N	Y	N	N	ND	N		Y	$3.16x10^{-4}$	Y	$3.16x10^{-4}$	Y	$3.16x10^{-5}$
CERM 11884			N	N	N	N	ND	N		N		N		N	
CERM 13061			N	Y	N	ND	ND	N		N		N		N	
Clozapine	5786-21-0		N	N	N	N	ND	N		N		N		N	
Corticosterone	50-22-6	Glucocorticoid	N	Y	ND	ND	ND	N		N		N		N	
Cortisol	50-23-7	Glucocorticoid	N	N	ND	N	ND	N		N		N		N	
Dehydroepian-drosterone	53-43-0	Androgen precursor	Y	Y	N	N	ND	N		N		N		N	
Dimethisterone	79-64-1	Progestin	N	Y	ND	N	ND	N		N		Y	$3.16x10^{-4}$	N	
Dopamine	62-31-7		N	N	N	N	ND	N		N		N		Y	$3.16x10^{-4}$
Erythromycin	114-07-8		N	N	N	N	ND	N		N		N		Y	$1.00x10^{-3}$
Ethacrynic acid	58-54-8		N	N	ND	ND	ND	N		N		N		Y	$1.00x10^{-6}$
Ethionine	67-21-0		Y	N	N	N	N	N		N		N		N	
Ferrous Sulphate	7782-63-0		N	N	ND	N	ND	N		N		N		N	
Fluoxymesterone without 17α-methyl	76-43-7	Androgen	N	Y	ND	ND	ND	N		N		N		N	
Flutamide	13311-84-7	Non-steroidal anti-androgen	N	N	N	N	ND	N		N		N		N	
Furafylline	80288-49-9		N	N	ND	ND	ND	N		N		N		Y	$1.00x10^{-3}$
Gentamicin A	1405-41-0		N	N	N	N	ND	N		N		N		N	
Indomethacin	53-86-1		N	N	N	N	N	N		N		N		N	
Iodoacetate	305-53-3		N	N	N	N	ND	N		N		Y	$1.00x10^{-3}$	Y	$1.00x10^{-5}$
Iproniazid	54-92-2		N	Y	N	ND	ND	N		N		N		N	
Isoprenaline	51-30-9		N	Y	ND	ND	ND	N		N		N		Y	$1.00x10^{-3}$

Table 6. Continued

Chemical	CAS number	Further information	Vitotox	Radar Screen	Ames	Mammalian gen. in vitro	Mammalian gen. in vivo	RAD51C luc	LEC (mol/l)	Cystatin A luc	LEC (mol/l)	p53 luc	LEC (mol/l)	Nrf2 luc	LEC (mol/l)
Ketoconazole	65277-42-1		N	N	N	ND	ND	N		N		Y	$1.00x10^{-3}$	N	
Labetalol	32780-64-6		N	N	N	ND	ND	N		N		N		N	
L-DOPA	79559-97-0		N	N	N	N	ND	N		N		N		Y	$1.00x10^{-4}$
Lilopristone with additional carbon in 17α chain		Progestin	Y(S9)	Y(S9)	N	ND	ND	N		N		Y	$1.00x10^{-3}$	N	
Methadone	76-99-3	N-Dialkyl	N	N	ND	N	ND	N		N		N		Y	$1.00x10^{-4}$
Naphazoline nitrate	5144-52-5		N	N	ND	ND	ND	N		N		N		Y	$3.16x10^{-4}$
N-ethylmaleimide	128-53-0	Thiol reactive	N	N	ND	ND	ND	N		N		N		Y	$1.00x10^{-5}$
Onapristone	96346-61-1	Anti-progestin	N	Y	N	N	N	N		N		Y	$1.00x10^{-3}$	N	
Org 10490			N	N	N	ND	N	N		N		N		Y	$3.16x10^{-4}$
Org 13011			N	N	N	ND	N	N		N		N		N	
Org 20091			N	N	N	N	ND	N		N		N		Y	$1.00x10^{-3}$
Org 20223			N	N	N	N	N	N		N		N		Y	$1.00x10^{-3}$
Org 20241	145261-31-0		N	N	N	ND	ND	N		N		N		N	
Org 20350			N	N	N	N	ND	N		N		N		Y	$3.16x10^{-4}$
Org 20660			N	Y(S9)	N	ND	ND	N		N		N		N	
Org 30189			N	N	N	ND	ND	N		Y	$3.16x10^{-5}$	N		Y	$1.00x10^{-5}$
Org 30535			N	N	N	N	ND	N		N		N		Y	$3.16x10^{-4}$
Org 30659	110072-15-6	Progestin	N	Y(S9)[a]	N	N	N	N		N		N		Y	$1.00x10^{-4}$
Org 30701			N	N	N	N	ND	N		N		N		N	
Org 31710	118968-41-5	Anti-progestin	N	Y	N	ND	N	N		N		N		N	
Org 32608			N	N	N	ND	ND	N		N		N		N	
Org 32782			N	Y	N	N	N	N		N		N		N	
Org 3362			N	N	N	ND	ND	N		N		N		N	
Org 34037	218899-99-1		N	N	N	N	ND	N		N		N		N	
Org 34694	160242-34-2	Estrogen	N	Y	N	N	N	N		N		N		N	
Org 34850	162607-84-3	Anti glucocorticoid	N	N	N	N	ND	N		N		N		N	
Org 34901	177900-66-2	Progestin	N	Y	N	N	ND	N		N		N		Y	$1.00x10^{-5}$
Org 37445		Estrogen	N	Y	N	ND	ND	N		N		N		Y	$1.00x10^{-3}$
Org 4060		Progestin	N	N	N	N	N	N		N		N		N	
Org 42788		Estrogen	N	Y	N	ND	ND	N		N		N		N	
Org 4428	135928-30-2		N	N	N	N	N	N		N		N		N	
Org 4874			N	N	N	ND	ND	N		N		N		N	
Org 5168			N	N	N	ND	ND	N		N		N		N	
Org 7258			N	Y	N	ND	ND	N		N		N		N	
Org 9340			N	Y	N	ND	ND	N		N		N		N	

Table 6. Continued

Chemical	CAS number	Further information	Vitotox	Radar Screen	Ames	Mammalian gen. in vitro	Mammalian gen. in vivo	RAD51C luc	LEC (mol/l)	Cystatin A luc	LEC (mol/l)	p53 luc	LEC (mol/l)	Nrf2 luc	LEC (mol/l)
Orphenadrine citrate	4682-36-4		N	N	ND	ND	ND	N		N		N		Y	$1.00x10^{-4}$
Papaverine HCl	61-25-6		N	N	ND	ND	ND	N		N		N		N	
Perhexiline	6724-53-4		N	N	N	ND	ND	N		N		N		N	
Perphenazine	58-39-9		N	N	ND	N	ND	N		N		N		N	
Phentolamine mesylate	50-60-2		N	N	N	N	ND	N		N		N		N	
Propylmesterolone	79243-67-7	Anti-estrogen	N	Y	N	N	N	N		N		N		N	
Quinidine	56-54-2		N	N	ND	ND	ND	N		N		N		N	
Quinidine sulfate	6591-63-5		N	N	ND	ND	ND	N		N		N		N	
R1881	965-93-5	Androgen	N	Y	ND	ND	ND	N		N		N		N	
Raloxifen	84449-90-1	SERM	N	Y	N	N	N	N		N		N		N	
Reserpine	50-55-5		N	N	N	N	ND	N		N		N		Y	$1.00x10^{-4}$
RU 58668	151555–47–4	Anti-Estrogen	N	Y	ND	ND	ND	N		N		Y	$1.00x10^{-3}$	Y	$3.16x10^{-4}$
Strychnine	57-24-9		N	N	ND	ND	ND	N		Y	$1.00x10^{-3}$	N		Y	$1.00x10^{-4}$
Sulfamoxole	729-99-7		N	N	ND	ND	ND	Y	$3.16x10^{-5}$	N		N		Y	$1.00x10^{-3}$
Sulfaphenazole	526-08-9		N	Y	ND	ND	ND	N		N		N		N	
Tularik 0191317		LXR/PXR agonist	N	N	ND	ND	ND	N		Y	$1.00x10^{-6}$	N		Y	$3.16x10^{-5}$
III. Equivocal for genotoxicity															
Digoxin	20830-75-5		N	N	Y/N	ND	ND	N		N		N		N	
Org 30002			N	N	Y/N	N	ND	N		N		N		Y	$1.00x10^{-3}$
Org 9217			N	N	Y/N	ND	ND	Y	$3.16x10^{-5}$	Y	$3.16x10^{-5}$	Y	$3.16x10^{-5}$	Y	$3.16x10^{-5}$
Org 9935			N	N	Y/N	N	ND	Y	$1.00x10^{-4}$	N		Y	$1.00x10^{-3}$	Y	$3.16x10^{-4}$

[a] compounds that are activated by S9 mixture but already show some activity without the addition of S9 mixture

Table 7. Number of scores in the reporter assays in each of the different groups from Tables 5 and 6

Group	RAD51C luc	Cystatin A luc	p53 luc	Nrf2 luc	N
I. Genotoxic compounds	19	34	44	67	108
i. Ames positive compounds	15	23	27	37	49
ii. Equivocal for Ames in vitro/in vivo mammalian genotoxic	1	1	1	1	1
iii. No Ames data or neg. in vitro/in vivo mammalian genotoxic	3	10	16	29	58
II. Not genotoxic or no genotoxicity reported	1	4	7	30	80
III. Equivocal for genotoxicity	2	1	2	3	4
Total	22	39	53	100	192

Validation of the HepG2 Cystatin A_luc reporter assay

In the Cystatin A_luc reporter assay, 39 of the 192 tested compounds (20%) compounds showed a positive result. As was observed in the p53_luc and RAD51C_luc reporter assays, most of the positive results in this assay were seen with genotoxic compounds (34/39).

Four compounds with a positive score were non-genotoxic or no genotoxicity data were reported. Like in the p53 reporter assay, bishydroxycoumarin had a positive result in this assay. Furthermore Org 30189, strychnine, and Tularik 0191317 had a positive result in the assay. Mammalian genotoxicity data was not available for Org 30189. With exception of the Nrf2_luc reporter assay this compound gave a negative result.

For both strychnine and Tularik 0191317, bacterial mutagenicity and mammalian genotoxicity data were lacking. With exception of Nrf2 induction, the compound showed no induction in the other assays.

Of the compounds with equivocal genotoxicity data, Org 9217 had a positive result in the Cystatin A_luc reporter assay.

Validation of the HepG2 p53_luc reporter assay

Of the 192 tested compounds, 53 (28%) had a positive score in the HepG2 p53_luc assay. Of which 44 compounds were genotoxic in one or more regulatory genotoxicity assays. For only seven compounds with a positive result, the regulatory genotoxicity data were negative or no genotoxicity was reported for these compounds. These results indicate that the specificity of this assay is high (44 scores in the group of 108 genotoxic compounds vs. 7 scores in the group of 81 compounds with negative genotoxicity data/ or compounds that lack genotoxicity data). This confirms the results that were found with the ECVAM compound list.

The seven compounds that might have a false-positive result were bishydroxycoumarin, dimethisterone, iodoacetate, ketoconazole, lilopristone (with an additional 17α carbon), onapristone, and RU58668. Bishydroxycoumarin was negative in the regulatory Ames, mammalian tests, and Vitotox™ assay. But this compound had a positive result in the

RadarScreen assay with a LEC of $1.00x10^{-4}$ M. In addition a positive result was observed in the Cystatin A_luc assay. As the Nrf2_luc assay showed a positive result, oxidative stress might be involved in this effect.

The progestin dimethisterone was not tested in the Ames assay and gave a negative result in the in vitro mammalian genotoxicity tests (no in vivo genotoxicity data). In the RadarScreen assay this compound showed a positive result, but it was negative in the RAD51C_luc, Cystatin A_luc, and Nrf2_luc assays. Besides this steroid, three other steroidal compounds were positive, i.e. the progestin lilopristone (with an additional 17α carbon), the anti-progestin onapristone, and the anti-estrogen RU 58668.

Lilopristone was reported to be negative in the Ames assay. Mammalian genotoxicity data were not available for this compound, but positive results were observed in the Vitotox™ and RadarScreen assay. This indicates that this compound may be genotoxic. On the other hand, the other reporter assays in the HepG2 cells showed negative results.

Onapristone showed a positive result in the RadarScreen assay, but it was negative in all the regulatory tests and other HepG2 reporter assays.

RU58668 lacks Ames and mammalian genotoxicity data. Furthermore, this compound had a positive result in the RadarScreen and Nrf2 reporter assays.

Surprisingly, iodoacetate gave a positive results in the p53_luc assay. With exception of the Nrf2_luc assay all other assays showed negative results for this compound.

Four compounds have equivocal results for genotoxicity. Of these, Org 9217 and Org 9935 had a positive result in the p53_luc reporter assay.

Validation of the Nrf2 reporter assay

Of the 192 tested compounds, 100 gave a response in the Nrf2 assay (52%). The percentage of compounds among the genotoxic compounds that had a positive response in the Nrf2 assay was 62% (67/108). In the group of non-genotoxins/compounds for which no genotoxicity data have been reported, the percentage of Nrf2 inducers was lower with 38% (30/80). These results confirm the observations with the ECVAM compound list. The percentage of Nrf2 positive compounds was especially high for bacterial mutagenic (positive Ames test) compounds with a score of 76% (37/49).

Of the four compounds that were equivocal for genotoxicity, Org 30002, Org 9217, and Org 9935 showed a positive response in the Nrf2 reporter assay.

Comparison of the reporter assays with the Ames assay, in vitro mammalian genotoxicity data and in vivo genotoxicity data

The results from the HepG2 luciferase reporter assays were compared with the results from the Ames assay and in vitro/in vivo mammalian genotoxicity assays (Tables 8-10). The HepG2 p53_luc assay had a correlation with the Ames assay of 73% (106/145).

The sensitivity was low with 55% (27/49), but the specificity was high with 82% (79/96). Similar results were observed with the HepG2 RAD51C_luc assay (sensitivity, 31%; specificity, 97%; correlation, 75%) and HepG2 Cystatin A_luc assay (sensitivity, 47%; specificity, 88%; correlation, 74%). When the three HepG2 reporter assay were combined the sensitivity was 61% (30/49). The specificity was high with 80% (77/96). This resulted in a correlation with the Ames test of 74% (107/145).

The results from the HepG2 reporter assays were also compared with the available in vitro mammalian genotoxicity data. The specificity of the HepG2 RAD51C_luc, Cystatin A_luc, and p53_luc reporter assays was high with scores of >83%. However, the sensitivity scores were low with 15% (12/78), 30% (23/78) and 40% (31/78) for the HepG2 RAD51C_luc, Cystatin A_luc and p53_luc assay, respectively. This resulted in a correlation with the in vitro mammalian genotoxicity scores for the HepG2 RAD51C_luc, Cystatin A_luc and p53_luc assay of 45% (56/124), 52% (65/124) and 56% (69/124), respectively. The overall correlation of the three reporter assays with in vitro mammalian genotoxicity was 59% (73/124). The sensitivity was 45% (35/78) and the specificity 83% (38/46). The correlation of the reporter assays with in vitro mammalian genotoxicity was relatively low compared to the correlation observed with the RadarScreen assay (76%).

The results were also compared with the in vivo genotoxicity data. For this comparison the sensitivity and predictivity were much better. The overall correlation of the three reporter assays with in vivo genotoxicity was 77% (54/70). Furthermore, the sensitivity and specificity were 74% (28/38) and 81% (26/32), respectively. For the individual assays, the correlations were 76% (53/70) for the HepG2 p53_luc assay (sensitivity, 71% (27/38); specificity, 81% (26/32)), 59% for the HepG2 RAD51C_luc assay (sensitivity, 29% (11/38); specificity, 94% (30/32)), and 76% (53/70) for the HepG2 Cystatin A_luc assay (sensitivity, 61% (23/38); specificity, 94% (30/32)). The overall correlation of the HepG2 reporters with in vivo genotoxicity was higher than the correlation that was measured with the RadarScreen assay (correlation 54% (37/69), sensitivity 68% (25/37), specificity 38% (12/32)). The in vitro mammalian genotoxicity tests detected almost all in vivo clastogenic compounds (sensitivity 94%), however, as expected the number of false positive results was high with a specificity score of only 46% (12/26).

Hierarchical clustering was also performed to show the similarity between the assays (Supplementary data, Figure 1; doi:10.1016/j/mrgentox.2009.12.007). The clustering was performed for a subset of compounds for which both in vitro and in vivo genotoxicity data were available. Hierarchical clustering shows that the HepG2 genotox reporters cluster with the in vivo genotoxicity assays and that the RadarScreen assay clusters with the in vitro mammalian genotoxicity assays.

Table 8. Comparison of the Vitotox™, RadarScreen, in vitro/in vivo mammalian genotoxicity and HepG2 reporter cell line data with the available Ames data. The sensitivity (sens), specificity (spec) and predictivity (pred) are shown.

	Vitotox™	RadarScreen	Mammalian genotoxicity		HepG2 genotox reporters				Nrf2_luc
			in vitro	in vivo	RAD51C	Cystatin A	p53	Total	
Sens(%)	86 (42/49)	55 (27/49)	83 (20/24)	73 (16/22)	31 (15/49)	47 (23/49)	55 (27/49)	61 (30/49)	76 (37/49)
Spec(%)	94 (90/96)	54 (51/95)	45 (34/75)	56 (22/39)	97 (93/96)	88 (84/96)	82 (79/96)	80 (77/96)	59 (57/96)
Pred(%)	91 (132/145)	54 (78/144)	55 (54/99)	62 (38/61)	75 (108/145)	74 (107/145)	73 (106/145)	74 (107/145)	65 (94/145)

Table 9. Comparison of the Vitotox™, RadarScreen, Ames, in vivo genotoxicity and HepG2 reporter cell line data with the available in vitro mammalian genotoxicity data. The sensitivity (sens), specificity (spec) and predictivity (pred) are shown.

	Vitotox™	RadarScreen	Ames	Genotoxicity	HepG2 genotox reporters				Nrf2_luc
				in vivo	RAD51C	Cystatin A	p53	Total	
Sens(%)	27 (21/78)	77 (59/77)	33 (20/61)	71 (34/48)	15 (12/78)	30 (23/78)	40 (31/78)	45 (35/78)	60 (47/78)
Spec(%)	89 (41/46)	74 (34/46)	90 (34/38)	87 (13/15)	96 (44/46)	91 (42/46)	83 (38/46)	83 (38/46)	61 (28/46)
Pred(%)	50 (62/124)	76 (93/123)	55 (54/99)	75 (47/63)	45 (56/124)	52 (65/124)	56 (69/124)	59 (73/124)	61 (75/124)

Table 10. Comparison of the Vitotox™, RadarScreen, Ames, in vitro mammalian genotoxicity and HepG2 reporter cell line data with the available in vivo genotoxicity data. The sensitivity (sens), specificity (spec) and predictivity (pred) are shown.

	Vitotox™	RadarScreen	Ames	Mammalian	HepG2 genotox reporters				Nrf2_luc
				gen. in vitro	RAD51C	Cystatin A	p53	Total	
Sens(%)	33 (13/38)	68 (25/37)	48 (16/33)	94 (34/36)	29 (11/38)	61 (23/38)	71 (27/38)	74 (28/38)	76 (29/38)
Spec(%)	78 (25/32)	38 (12/32)	79 (22/28)	46 (12/26)	94 (30/32)	94 (30/32)	81 (26/32)	81 (26/32)	66 (21/32)
Pred(%)	54 (38/70)	54 (37/69)	62 (38/61)	74 (46/62)	59 (41/70)	76 (53/70)	76 (53/70)	77 (54/70)	71 (50/70)

Risk analysis of genotoxicity in the early research phase of drug development by using the in vitro assays and hierarchical clustering

We evaluated six different mechanism-based in vitro assays that can help to detect genotoxic compounds. The data from all these compounds (with exception of mestranol) were hierarchically clustered. After clustering the available regulatory genotoxicity data was added to the list (Supplementary data, Figure 2; doi:10.1016/j/mrgentox.2009.12.007). In case of the ECVAM compounds this data was not added as for a lot of these compounds in vitro data is positive however irrelevant due to cytotoxicity or another mechanism of action. Performing hierarchical clustering makes it possible to easily detect compounds that show species specific genotoxicity and to detect structural components in compounds that might cause genotoxicity.

Most of the compounds that were negative in all the assays were also negative in the reported regulatory genotoxicity data. Exceptions were tamoxifen, ment-bucyclate, hydrochlorothiazide, dexamethasone and carbon tetrachloride. For other compounds only Nrf2 activity was observed.

Clustering of the compounds also shows that most compounds that are strong genotoxicants like for example MMS, cisplatin and ENU give a positive response in most or all of the assays. Compounds between number 93 and 105 in Supplementary data showed only activity in the Vitotox™ assay. Most of these compounds are structurally related. Similarly there were quite a few compounds that showed only activity in the RadarScreen assay (± Nrf2 activity). Many of these are steroids, which stresses the high sensitivity of this assay for this structure class. In in vitro mammalian genotoxicity tests positive results have been reported for these or structurally related compounds. In vivo most of these compounds show a negative result. In addition, several of these RadarScreen positive compounds are genotoxic compounds that need activation by CYP2B6 and CYP2E1, which are not detected in HepG2 cells.

The RAD51C_luc assay focuses on a specific mechanism. In almost all cases where RAD51C activation was observed, p53 was also induced (31/37). In the case of curcumin, 4NQO and Org 5695 a reproducible tendency for induction between 1.3 and 1.5 fold was observed. The same was true for Cystatin A as most of the compounds that showed a positive response in the Cystatin A assay were also positive in the p53_luc assay, or showed a tendency to induction in the p53_luc assay.

Discussion

The reporter assays

In the present study, four mechanism-based luciferase reporter assays were developed. Reporter assays with a p53- and Nrf2-responsive element have already been described by others. However, the number of compounds that were used for the evaluation of these assays were much smaller.

Reporter assays involving p53 have been developed by Ohno et al. [33, 34] and Sohn et al. [35]. Both studies describe the development of a stable cell line transfected with a luciferase based p53 reporter system. Like in the study performed by Sohn et al., we used a construct with four copies of the 10-bp motif: 5'-PuPuPuC/(A/T)(T/A) GPyPyPy-3'. Ohno et al. used a slightly different sequence. They used a small part of the p53R2 promoter. Their reporter vector finally contained six times the 10-bp motif. We cannot exclude that this difference can result in a different test outcome. Moreover, the application of different cell lines might cause differences in responses.

Sohn et al. develop their assay in the human pancreatic carcinoma cell line HS776T and showed that p53 was activated by alkylating agents, intercalating agents, anti-metabolites, topoisomerase inhibitors and anti-microtubule agents. In their assay cyclophosphamide did like in the present study not induce p53.

Ohno et al. made their assay in the human breast adenocarcinoma cell line MCF-7 and HepG2 cells. They found that MCF-7 cells were slightly more sensitive than HepG2 cells, and validated their assays with 80 chemicals [34]. Data were compared with data from the Ames assay and data from other genotoxicity assays including the mouse lymphoma assay, chromosome aberration test, sister chromatid exchange (SCE) assay, and in vivo carcinogenicity data from rats or mice. Forty eight chemicals were positive in their assay. Of these, 40 were positive in the Ames test. The eight remaining compounds were all positive in other genotoxicity tests, and seven were carcinogenic in mice or rats. This indicates that a p53 reporter assay may indeed have additional value compared to the Ames test. Furthermore the addition of S9 mixture was tested by Ohno et al. [34]. They could trigger the induction of some compounds. However, the six compounds that needed S9 mixture for a positive result in the Ames test already showed a positive result in the p53-reporter assay without S9 mixture indicating that metabolism was present in HepG2 (and MCF-7) cells. Three mutagenic (Ames positive) compounds were negative in the p53 reporter assay. Surprisingly, one of these compounds was 5-fluorouracil. This compound was clearly positive in the present assay and in the assay described by Sohn et al [35].

The insert in the Nrf2 reporter assay was based on the study of Boerboom et al. [36]. A functional ARE sequence of the human NQO1 gene was placed in a pGL3 vector. A study of Wang et al. [37] showed that placing more AREs before the luciferase gene can

increase the level of induction. For this reason, we prepared a vector with a single ARE and a vector with three ARE repeats. Experiments using transient transfection did not show any difference in the induction of the luminescence between these two constructs. The same was observed for stably transfected cell pools (Schering-Plough, unpublished data). In the end, a single cell clone with a single ARE showed the largest induction of the luminescence. This cell clone was used for the experiments.

Luciferase reporters driven by the RAD51C and Cystatin A promoter were also generated. As far as we know it is the first time that results from a RAD51C and Cystatin A reporter assay are presented. RAD51C was chosen as this gene plays an important role in double strand break repair.

The role of the anti-apoptotic protein Cystatin A in relation to genotoxicity is less clear. The results in this study showed that induction of Cystatin A is quite specific for genotoxicity. However, also bile acids can induce this gene [21]. The induction found in this study with the LXR agonist Tularik 0191317 may be related to this effect. Thus some non-genotoxic compounds might induce Cystatin A.

The assays developed in the present study have a sensitive luminescence read-out. An advantage of this system over a more common used fluorescent read-out is that autofluorescence of compounds does not hamper genotoxicity assessment [38]. Furthermore a sensitive assay makes the assessment of a NOEL (no observed effect level) more accurate which is very important for proper risk assessment.

Validation of the assays with the ECVAM compound list

A compound list published by the ECVAM was used for the evaluation of the four reporter assays [28]. The sensitivity of the HepG2 p53_luc, RAD51C_luc, Cystatin A_luc assays was 85% (17/20), 60% (12/20) and 70% (14/20), respectively. The fact that the RAD51C reporter assay measures the induction of a more specific mechanism than induction of for example p53, was the reason for the lower sensitivity of this assay. An advantage of measuring the induction of such a specific mechanism is the mechanism-based insight and the high specificity of such an assay.

The metabolic capacity present in the HepG2 cell line was sufficient to activate several proximate genotoxins such as benzo[a]pyrene, 7,12-dimethylbenzanthracene, 2-acetylaminofluorene, 2,4-diaminotoluene, IQ, PhIP.HCl and aflatoxin B1. All these compounds showed a positive result in the p53_luc assay without the addition of S9 mixture. Not all compounds that needed metabolic activation were positive in the p53_luc assay. Cyclophosphamide and dimethylnitrosamine showed a negative results. As CYP2B6 and CYP2E1 are expressed at a very low levels in the HepG2 cells [28] this is a plausible reason for the fact that these compounds were not detected. These two compounds were detected in the RadarScreen assay and they showed the highest activity in this assay after the addition of S9 mixture [1]. Besides these two compounds,

chloramphenicol was not detected in the p53_luc assay.

Induction in the RAD51C_luc assay was found for 12 of the 20 compounds showing that double strand break repair is involved in the genotoxic mode of action of these compounds. Of the compounds positive in the p53_luc assay PhIP.HCl, cadmium chloride, cisplatin and p-chloroaniline did not induce the luminescence in the RAD51C_luc assay. In addition, as seen in the p53_luc assay, cyclophosphamide, dimethylnitrosamine and chloramphenicol were negative in the RAD51C_luc assay.

The same compounds were also not detected in the Cystatin A_luc assay. Activity was also not measured for PhIP.HCl, cadmium chloride and p-chloroaniline.

When the HepG2 genotoxicity reporter assays were combined the sensitivity was similar to that found for the p53_luc assay, i.e. 85% (17/20). Of the 20 genotoxic compounds 75% (15/20) showed activation of the Nrf2 pathway.

Application of a pre-screen that consists of a Vitotox™ assay, RadarScreen assay and the three HepG2 genotoxicity reporters would result in a sensitivity of 95% (19/20).

The three HepG2 genotoxicity reporter assays showed all a specificity that was >90%. Of the 23 compounds that were defined by the ECVAM as non-DNA reactive chemicals fluometron and phenanthrene showed a positive response in the p53_luc assay. Both compounds also caused oxidative stress. Fluometron showed a negative response in the remaining assays. Phenanthrene was positive in the Vitotox™ assay after the addition of S9 mixture. The effects of these two compounds were only observed at the highest test dose of $1.00x10^{-3}$ M indicating that the effects are only small. Ampicillin was positive in the RAD51C_luc assay and showed a tendency to induction in the Cystatin A_luc assay. Moreover, a positive response in the RadarScreen was observed in the presence of S9. Of the 23 non DNA-reactive compounds, five showed a positive response in the Nrf2_luc assay.

For two of the ECVAM compounds that are in the 'false positive group', i.e. tertiary-butylhydroquinone and curcumin, there is still discussion within the ECVAM working group whether these compounds are real genotoxic positives as they give rise to oxidative stress which can result in DNA damage (personal communication with David Kirkland). In both the Vitotox™ and RadarScreen assay tertiary-butylhydroquinone gave a positive response. In the HepG2 Nrf2_luc reporter assay it was shown that this compound indeed caused oxidative stress with a LEC of $1.00x10^{-5}$ M. In the other three reporter assays only an induction was observed in the Cystatin A_luc assay. Overall, the six assays show that this compound might indeed be genotoxic. The same is true for curcumin. Although this compound was negative in the Vitotox™ assay, it showed a positive result in the RadarScreen assay. In HepG2 cells this compound showed Nrf2 activation and an induction of double strand break repair. Moreover, a tendency for induction (just below 1.5 fold) was observed in the HepG2 p53_luc assay. Overall, these results indicate that curcumin indeed might be a genotoxic compound.

Of the other compounds in the 'false positives group', propyl gallate was positive in the four HepG2 reporter assays. None of the other compounds were positive in the HepG2 p53_luc assay. Fluometron and phenanthrene were positive in the p53_luc assay. Besides the above mentioned compounds, urea showed a positive response in the Cystatin A_luc assay.

Induction of the luminescence in the Nrf2_luc assay was observed for 8 of the 19 compounds in the 'false positives group'. Overall this resulted in 31% positive scores (13/42) for the 42 non-genotoxic compounds. This percentage is much lower than the percentage of 75% that was observed with the genotoxic compounds. This may indicate that the number of compounds that cause oxidative stress is higher under the genotoxic compounds.

However, cross-talk between the p53 and Nrf2 pathways makes interpretation of data more difficult. Recently Chen et al. [39] showed that p21 activates Nrf2 (p21 is activated by p53). On the other hand Faraonio et al. [40] showed that p53 suppresses Nrf2. However, these effects are suggested to occur only at such high levels of stress were damage is irreparable. This shifts the stress response towards cell death (apoptosis). Moreover, oxidative stress can of course result in activation of the Nrf2 response. At higher levels of stress, DNA damage and p53 activation can then occur. Because of the crosstalk between these two pathways it is difficult so not impossible to delineate cause and effect. The only conclusions that can be drawn is that genotoxicants show a high incidence of activation of the Nrf2 pathway.

When the three genotoxicity reporter assays are combined the specificity decreases but is still 81% (34/42). Addition of the Vitotox™ and RadarScreen assay data to this pre-screen would result in a specificity of 74% (31/42). These values result in an overall predictivity of the HepG2 reporter assays of 82% (51/62). The predictivity of a screen using all five genotoxicity reporter assays is 81% (50/62).

Validation of the assays with an additional set of compounds including a large set of steroidal compounds.

Besides the chemicals of the ECVAM list, an additional set of 192 compounds were tested. These were non-genotoxins and genotoxins with diverse mechanisms of action. However, for a large number of these compounds the mechanism is still unknown and the list also included compounds from which no genotoxicity data are available. The list also included a lot of steroidal compounds. In addition to progesterone, which was in the ECVAM compound list, several other steroidal compounds including estrogens, anti-estrogens, progestins, anti-progestins, androgens, glucocorticoids and anti-glucocorticoids were tested.

Estrogens are carcinogenic in humans and rodents [41, 42]. Epigenetic mechanisms that stimulate cell proliferation through the estrogen receptor are thought to be the

main factors that contribute to this effect. Genotoxic properties of estrogens may also contribute to this [43]. However, most genotoxic effects are observed at concentrations that are >1000-fold above the physiological concentration of 100 pM. During pregnancy the estrogens levels can reach a concentration of 10 nM. However, the physiological relevance of the reported genotoxic effects remains questionable. Only for catechol estrogens positive results for bacterial mutagenicity were demonstrated [44]. The other positive results were found in mammalian genotoxicity tests. Catechol estrogens are reported to be converted easily by various oxidation mechanisms to ortho-quinones [45]. These ortho-quinones react with all DNA bases with exception of thymidine. The six catechol estrogens showed a positive result in the p53 and Nrf2 reporter assays. None of these compounds seemed to induce double strand breaks as no induction of the luminescence was reported in the RAD51C_luc reporter assay. Moreover ,with exception of 2-hydroxy-estradiol and 2-methoxy-estradiol a positive result in the Cystatin A assay was reported.

Besides the catechol estrogens, 16 other estrogens were tested. Of these compounds 16α-hydroxy-estrone, diethylstilbestrol, equilin, equilin-7α-methyl, estradiol-17α, estradiol-17β, ethinylestradiol-17β and mestranol showed a positive result in the p53 reporter assay. All these steroidal and stilbene estrogens contain an aromatic A-ring with a free hydroxyl group. The 2-hydroxylation or 4-hydroxylation of these compounds makes the formation of catechol estrogens possible [46]. Estrogens without an aromatic A ring like Org 34694, Org 37445, en Org 42788 did not show genotoxicity in the present study. Of these, Org 34694 has a $\Delta^{4(5)}$ double bond, while Org 37445 and Org 42788 contain a $\Delta^{5(10)}$ double bond. Due to their conformation these compounds have estrogenic activity, but they lack the ability to form catechols. Therefore these estrogens may be less hazardous with respect to genotoxicity. Not all estrogens with an aromatic A ring and free OH group induced p53; estrone and estriol did not show these effects. Experiments performed by Yagi et al. [43], also showed that estrone induced no DNA damage. The four anti-estrogens (also without an aromatic A ring) do not induce genotoxicity in HepG2 cells.

None of the tested androgens showed genotoxicity in the three HepG2 genotoxicity reporter assays. Generally androgens lack bacterial mutagenicity and mammalian genotoxicity in the regulatory tests [44]. Only for fluoxymesterone a weak genotoxic potential has been reported [47].

Of the 21 tested progestins and anti-progestins only dimethysterone, lilopristone (with additional carbon in 17α chain) and onapristone showed a positive response. Onapristone and lilopristone contain a N-dialkyl group, which has been reported to be a structural property of many compounds that intercalate in the DNA [48]. This structural group may be the reason for the positive effect. However, not all tested compounds with a N-dialkyl group showed genotoxicity in the reporter assays in the HepG2 cells. Compounds like

for example chlorpromazine, chlorprothixene and imipramine did not show such an effect. Onapristone and dimethysterone were negative in the regulatory tests that were performed. Lilopristone with additional carbon in 17α chain, was not tested in the regulatory genotoxicity tests, but lilopristone was negative in these tests [44]. Progestins can be divided into the norethisterone, norgestrel, spirolactone and medroxyprogesterone group. The majority of studies with progestins from the first three groups produced negative results in the regulatory genotoxicity assays. For several progestins from the medroxyprogesterone group, positive results have been described [44] Progestins in this group share the 17-hydroxy-3-oxo-pregna-4,6-diene structure. It has been shown for cyproterone acetate that a highly reactive carbonium ion is formed following reduction of the 3-keto group and sulfoconjugation [44]. The genotoxic potency was related to the capacity of metabolization through these pathways. However these reactions do not take place in the routine regulatory genotoxicity tests. The kidney of the hamster is a place where these reactions take place. In the HepG2 cells none of the tested progestins in this group showed a positive response.

Glucocorticoids were reported to be negative in the Ames and mammalian genotoxicity tests. An exception is dexamethasone, for which positive results have been reported. In the reporter assays, dexamethasone and the other tested corticosteroids corticosterone, cortisol and anti-glucocorticoid Org 34850 showed no genotoxicity. The positive results that were found with dexamethasone were suggested to be false-positive due to interpretation of micronucleus assay data, as dexamethasone causes the formation of apoptotic bodies.

Overall, for many steroids genotoxicity has been reported. But in most cases positive as well as negative responses have been reported for these compounds. The assays in the present study give the indication that like reported in literature catechol estrogens and estrogens from which these catechols can be formed give the highest risk on genotoxicity. The assays described in the present study might help to develop non-genotoxic estrogens.

Besides the steroidal compounds, the list of 192 compounds also contained other compounds. Importantly, the two aneugens in this list being colchicine and noscapine HCl were genotoxic in the HepG2 cells. Since taxol (ECVAM list) was also genotoxic in HepG2 cells, aneugenic compounds appear to be detected in the reporter assays. This is an advantage of these reporter assays, as these compounds were not detected in the RadarScreen assay. Moreover, topoisomerase II inhibitors, intercalating compounds, anti-metabolites and compounds causing oxidative stress could be detected in HepG2 cells.

Comparison of the reporter cell line results with bacterial mutagenicity (Ames test) in vitro and in vivo mammalian genotoxicity data

The results obtained with the 192 additional compounds were compared with the available bacterial mutagenicity data, in vitro mammalian genotoxicity data, and in vivo genotoxicity data. The correlation with bacterial mutagenicity was 74% (sensitivity, 61%, 30/49; specificity, 80%, 77/96). Besides Ames positive compounds, Ames negative clastogenic compounds were detected. However, the correlation between the results from the HepG2 reporter assays and in vitro mammalian genotoxicity was relatively low with only 59% (sensitivity, 45%, 35/78; specificity 83%, 38/46). This score is lower than the predictivity of 76% (sensitivity, 77%, 59/77; specificity 74%, 34/46) that was previously observed for the RadarScreen assay. However, the correlation between results from the HepG2 genotoxicity reporter assays and in vivo genotoxicity was much higher with 77% (sensitivity, 74%, 28/38; specificity 81%, 26/32). In case of in vivo genotoxicity the predictivity of the RadarScreen was only 54% (sensitivity, 68%, 25/37; specificity 38%, 12/32). These results show that the HepG2 cells have a better correlation with in vivo genotoxicity than with in vitro mammalian genotoxicity data. This was confirmed by using hierarchical clustering. The presence of phase II metabolism and a functionally active p53 protein might be the reason for these scores.

Recently a GADD45a-GFP assay in the human lymphoblastoid cell line TK6 (GreenScreen HC, Gentronix) was developed by Hastwell et al. [49]. This assay had a high sensitivity and specificity of respectively 71% and 100% for in vivo genotoxicity. With a different compound set Olaharski et al. [50] showed that the sensitivity of this assay dropped to only 30% (comparison with in vitro mammalian genotoxicity). However, the specificity was still high with 97%. In response to this study, Walmsley and Billinton reanalyzed the data [51]. Given the fact that in vitro genotoxicity tests give often false positive results, a carcinogen with positive in vitro mammalian genotoxicity data may still be a non-genotoxic carcinogen. Hastwell et al. tried to make a better classification of genotoxic carcinogens and non-genotoxic carcinogens. In their analysis the sensitivity of the GADD45 assay increased to 75%. However, the compound set became smaller. Moreover, as stated by Olaharski et al. such an analysis of the data might also introduce subjectivity. However, the importance of the comments made by Walmsley et al. surely is that in vitro mammalian genotoxicity tests give often false positive results and that this does not always reflect the classification of genotoxic/non-genotoxic carcinogenicity.

Therefore, it is in our view better to compare data from our HepG2 reporter assays to the in vivo genotoxicity tests when a prediction for (genotoxic) carcinogens is made.

Hierarchical clustering of the compounds

The results obtained with all compounds were ranked by hierarchical clustering. Doing this makes it more easy to detect species specific genotoxicity and structural components of compounds that may cause genotoxicity. Clustering of the compounds showed that most compounds that are strong genotoxicants give a positive response in most of the assays. Performing all six assays in parallel makes it possible to perform risk assessment at an early time point during the development of new drugs. Results from newly tested compounds can be compared with the pattern and structure of compounds that have already been tested. For doing this, it is very important to know the weaknesses of the assays. For example compounds that need metabolic activation by CYP2B6 and CYP2E1 are probably missed in the HepG2 reporters and aneugens are not detected in the RadarScreen assay. Furthermore, in the Vitotox™ assay mutagenic (Ames positive) compounds such as melphalan and cyclophosphamide, which have a $N(CH_2\text{-}CH_2\text{-}Cl)_2$ group, are missed.

Conclusion

We have developed four mechanism-based reporter assays in the HepG2 cell line, which can be applied for screening in the research phase of drug development. In combination with the previously validated Vitotox™ and RadarScreen assays it seems that a good prediction can be made for bacterial mutagenicity (Ames results), in vitro mammalian genotoxicity, and in vivo genotoxicity. Application of these assays in the research phase of drug development can visualize problems at an early stage before compounds enter the regulatory tests.

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Chapter 8

Pharmacologic profiling of human and rat cytochrome P450 1A1 and 1A2 induction and competition

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Abstract

Strong activation of the AhR can lead to various toxic effects such as (non-genotoxic) carcinogenicity. Moreover, drug-drug interactions by (non-)competitive inhibition of CYP1A1 and 1A2 may cause adverse side-effects. Normally the majority of toxicity studies are performed in rats, while for the prediction of human toxicity human AhR activation and CYP1A competition should be studied. The present study focused on the deselection of strong AhR activators and/or CYP1A inducers and (non-)competitive inhibitors in the early phase of drug development, as well as on species differences between humans and rats.

Induction studies were performed in the human HepG2 and rat H4IIE cell lines. A set of 119 compounds, including known AhR ligands were tested. CYP1A induction was observed for 24 compounds. In H4IIE cells, more compounds showed induction and most EC50 values were below those of HepG2 cells. Species specific CYP1A induction in H4IIE and HepG2 cells was obtained for eight and three compounds, respectively.

The same compounds except four in-house NCEs were used to study differences between CYP1A1 and 1A2 competition in human and rat supersomes. Of the 115 compounds, 46 showed CYP1A1 competition. Competition was human and rat specific for 12 and 10 compounds, respectively. CYP1A2 competition was observed for 37 compounds of which 14 and 3 compounds showed human and rat specific inhibition, respectively.

In conclusion, for several compounds species differences between CYP1A induction and competition in human and rat were found. Therefore, parallel screening in both species may be a useful strategy for toxicity screening in the early discovery phase of drug development.

Introduction

In drug therapy, cytochrome P450 (CYP) 1A induction may lead to drug-drug interactions and toxic side effects. The regulation of CYP1A is mainly aryl hydrocarbon receptor mediated (AhR). The AhR was discovered as the receptor that binds the environmental contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) [1]. The AhR is a basic helix-loop-helix protein belonging to the Per-ARNT-Sim (PAS) family [2] and is located in the cytoplasm in an inactive complex with Hsp90 and p23. Experiments in Hsp90 deficient yeast show that Hsp90 is needed for gene induction by the AhR [3, 4] Hsp90 is thought to protect AhR from degradation and to stabilize the high-affinity ligand-binding conformation of the AhR [5]. Binding of a ligand to the AhR leads to activation of the receptor and subsequently in translocation to the nucleus. In the nucleus AhR releases its partner Hsp90 and forms a heterodimer with the AhR nuclear translocator protein (ARNT). Further activation of this heterodimer by phosphorylation and/or dephosphorylation is required for DNA binding [6, 7].

Strong activation of the AhR by TCDD results in toxic effects like a wasting syndrome, thymic involution, endocrine disorders, and very important teratogenicity and (non-genotoxic) carcinogenicity. Mortality studies involving occupational exposure to TCDD have demonstrated an increased risk for several types of cancer in humans [8-10]. Important for this non-genotoxic carcinogenic effect might be the change in expression patterns of several factors that are involved in cellular growth and differentiation. Another effect contributing to carcinogenicity might be the impairment of the p53 response [11, 12].

Studies in AhR-null mice have been used to investigate the role of the AhR in mediating the toxic effects. These studies revealed that AhR-null mice were resistant to TCDD induced lesions, strongly suggesting that the toxic effects are mediated by the AhR [13]. Furthermore, skin tumors that appear after topical application of the AhR agonist benzo[a] pyrene (B[a]P) are absent in AhR-null mice [14]. Besides activating the AhR, B[a]P is metabolized by CYP1A1 to a genotoxic metabolite [15]. The metabolic activation of proximate carcinogens to reactive metabolites by CYP1A1 and 1A2 was also shown for other AhR agonists by using Aroclor pretreated rat liver S9 mixture for metabolic activation in the Ames test. CYP1A1 and/or 1A2 activates PAHs, nitrosamines and aryl amines into reactive metabolites that induce mutations [16]. Induction of CYP1A enzyme activity is often used for the detection of AhR ligands as good specific CYP1A substrates are available.

Besides metabolic activation into genotoxic compounds, drug-drug interactions caused by (non-)competitive inhibition of CYP1A1 and 1A2 can also lead to adverse side-effects. Many drugs are metabolized by CYP1A2 and therefore drug-drug interactions

caused by CYP1A2 inhibition are known. CYP1A2 constitutes about 13% of the total hepatic CYP content and is one of the clinically most relevant CYP isoenzymes. Together with CYP2C9, 2C19, 2D6, and 3A4 it performs the main part of drug metabolism. Examples of drugs metabolized by CYP1A2 are the antidepressants amitriptyline HCl, clomipramine HCl, and desipramine HCl [17-19]. The levels of CYP1A1 in the human liver are low, however, this enzyme is highly inducible in the liver and extra hepatic tissues. Induction of CYP1A1 by AhR agonists in human precision-cut liver slices and primary human hepatocytes has been shown in several studies [20-23]. Little is known about drug-drug interactions in which CYP1A1 is involved. However, some of the drug-drug interactions caused by ketoconazole might be the result of a lower CYP1A1 enzyme activity caused by inhibition or competition in the intestine [24]. Furthermore, synergistic embryotoxicity of AhR agonists with CYP1A1 inhibitors has been shown [25]. This was probably due to a prolonged activation of the AhR.

Because of the adverse side-effects it might be useful to screen for and deselect candidate drugs that are strong AhR activators and/or strong CYP1A1 and 1A2 inhibitors/competitors in the early phase of drug development.

Induction of CYP1A enzyme activity was measured with the fluorogenic substrate 3-Cyano-7-ethoxycoumarin (CEC) and luminogenic P450-glo substrate Luciferin-CEE (Luc-CEE). The latter substrate is converted by CYP1A1 into luciferin, which in turn reacts with luciferase to produce light that is directly proportional to the activity of CYP1A1.

Because species differences have been reported between CYP1A induction in rats and humans [26], the CEC and the P450-glo 1A1 induction assays were performed in the human HepG2 as well as the rat H4IIE cell line.

CYP1A1 and 1A2 inhibition were measured in a 384-well high-throughput assay using CYP1A1 and 1A2 expressing supersomes and CEC as fluorogenic substrate. With these assays competition was measured between the fluorogenic substrate CEC and the tested compounds for both CYP1A1 and 1A2. Whether compounds are non-competitive or competitive CYP1A1/1A2 inhibitors cannot be determined with these assays. Moreover, in order to study species differences, competition experiments were performed by using both human and rat supersomes. A total of approximately 119 compounds were tested in the CYP1A induction and competition assays. These compounds included narcotic analgesics, hypnotics, vasodilators, specific cellular energy blockers, cellular proliferation inhibitors, ion channel blockers, estrogens, antiestrogens, androgens, progestagens, polychlorinated biphenyls (PCBs), and others.

Materials and methods

Materials

All compounds and reagents were of analytical grade. Polychlorinated biphenyls including:

2,3',4,4'-tetrachlorobiphenyl (PCB 77), 2,3,3',4,4'-pentachlorobiphenyl (PCB 105), 2,3,4,4',5 pentachlorobiphenyl (PCB 114), 2,3',4,4',5'-pentachlorobiphenyl (PCB 118), 2,3',4,4',5'-pentachlorobiphenyl(PCB123),3,3',4,4',5'-pentachlorobiphenyl(PCB126), 2,3,3',4,4',5-hexachlorobiphenyl (PCB 156), 2,3,3',4,4',5'-hexachlorobiphenyl (PCB 157), 2,3',4,4',5,5'-hexachlorobiphenyl (PCB 167), 3,3',4,4',5,5'-hexachlorobiphenyl (PCB 169), 2,3,3',4,4',5,5'-heptachlorobiphenyl (PCB 189), and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) were purchased from Promochem (Wesel, Germany).

All other compounds were from Sigma-Aldrich (St. Louis, USA). Luciferin-CEE (Luc-CEE) was from Promega (Madison, USA). CEC and supersomes expressing human and rat CYP1A1 and 1A2 were from BD Biosciences (San Jose, USA). Trypsin and Dulbecco's Modified Eagles medium, Nutrient Mixture F-12 (DMEM/HAM F12 medium in a ratio of 1:1) without phenol red was from Invitrogen (Kalsruhe, Germany), defined supplemented bovine calf serum (dBCS) from Hyclone (Utah, USA) and white 96- and 384-well culture plates from Perkin Elmer (Groningen, The Netherlands).

Cell culture

HepG2 and H4IIE cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured in Dulbecco's Modified Eagles medium and Nutrient mixture F-12 mixed in a ratio of 1:1 with 10% dBCS and 1% Penicillin-Streptomycin (10,000 U/ml, Invitrogen). Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37 °C and medium was refreshed every 3 or 4 days with subculturing.

Preparation of compound solutions

Stock solutions of 0.1 M were dissolved in 100% dimethyl sulfoxide (DMSO). The stock solution of TCDD had a concentration of $3.16x10^{-4}$ M. From the stock, √10 dilutions were prepared in DMSO.

Test plate preparation induction assays

HepG2 and H4IIE cells were trypsinized, counted and seeded in 96-well plates. HepG2 and H4IIE cells were resuspended in culture medium to a final concentration of $3x10^4$ and $2x10^4$ cells/well, respectively. The 96-well microtiter plates were incubated for 24 h in a humidified atmosphere at 37°C under 5% CO_2. Following the incubation, 11 serial

culture medium dilutions of the compounds or a control sample were added as 10 µl fractions to HepG2 and H4IIE cells leading to a final volume of 200 µl. The highest tested concentration for the compounds was 10^{-4} M. An exception was TCDD for which the highest tested concentration was $3.16x10^{-7}$ M. The final concentration of DMSO in the assays was 0.1 %.

CEC CYP1A induction assay

The induction of CYP1A activity in HepG2 and H4IIE cells was measured with the fluorogenic substrate CEC. CEC reacts with both CYP1A1 and 1A2. After incubation of cells with compounds for 24 h, cells were washed twice with PBS. Next 100 µl of 40 µM CEC dissolved in culture medium without dBCS was added. After 30 min the fluorescent signal was read on the Victor II (Perkin Elmer) by means of excitation at 409 nm and emission measurement at 460 nm.

P450-Glo CYP1A1 induction assay

The induction of CYP1A1 activity in HepG2 and H4IIE cells was measured with the luminogenic substrate Luc-CEE. After incubation of cells with compounds for 24 h, HepG2 and H4IIE cells were washed twice with PBS and the CYP1A1 induction was assessed with Luc-CEE. To the plates 50 µl Luc-CEE (30 µM) was added. Subsequently, 50 µl luciferin detection reagent was added. Plates were shaken for 10 min and the luminescence signal was measured with a TopCountNT luminometer (Perkin-Elmer).

Cytochrome P450 1A1 and 1A2 competition assays

CYP1A1 and 1A2 competition assays were carried out by using supersomes. Compounds were tested at concentrations of 10^{-7} M to 10^{-4} M with √10-fold dilution steps in a 384-well plate in 0.1% DMSO. The highest concentration TCDD in the assay was $3.16x10^{-7}$ M. The cofactor solution was prepared in 25 mM phosphate buffer (pH 7.4) and contained 2.6 mM $NADP^{+}$, 6.6 mM glucose-6-phosphate, 6.6 mM $MgCl_2$ and 0.8 U/ml glucose-6-phosphate dehydrogenase. This solution was prewarmed at 37ºC. Five minutes before the start of the reaction, human or rat CYP1A1 or 1A2 supersomes were added to the cofactor solution leading to a concentration of 5 pmol/ml supersomes in the final reaction. To the 384-well plates, containing 10 µl compound dilution or DMSO control, 10 µl of a substrate solution containing 20 µM CEC in 325 mM phosphate buffer was added. The plates were covered with a lid and shaken for 20 min. Then plates were pre-warmed at 37ºC in an incubator and 20 µl of an enzyme/cofactor solution was added leading to a final volume of 40 µl/well. Thereafter the plate was put in the Victor II reader, shaken for 20 s and pre-incubated for 2 min at 37ºC. Next the fluorescence was measured (t=0 min). The excitation wavelength was set at 409 nm and the emission was measured at 460 nm. Then the plate was incubated for 30 min at 37ºC. Thereafter

the fluorescence was measured (t=30 min). The difference in fluorescence between the measurement at 0 and 30 min was used for calculation of CYP competition. The increase in fluorescence of the solvent control (0.1% DMSO) during these 30 min was set at 100% activity. For calculation of the IC50 values of competitors, the data were logit transformed. The software program Xlfit (version 4.1, ID business solutions limited) was used for the calculation of the best line fitting the experimental data. From this line the IC50 was determined. The efficacy was defined in percentage by means of the total inhibition of the fluorescence increase at the highest tested compound concentration. This concentration was 10^{-4} M, with exception for TCDD for which the highest concentration tested was $3.16x10^{-7}$ M.

Statistical analysis

Each experiment was performed in duplicate in three independent experiments. Data analysis was performed by using a Student's *t* test. This indicated that an induction above control level of 20% and 50% for the CEC and P450-glo assay, respectively, was statistical significant. For the assessment of this threshold 5 independent experiments were performed with 3-methylcholanthrene, indirubin, and indigo (data not shown). Since compounds with an induction factor equal to or greater than 2-fold are defined as inducers this is based on statistically significant differences. Likewise 5 independent competition assays were carried out with furafylline and ketoconazole. The Student's *t* test showed that an inhibition of 20% was statistically significant. Therefore the IC50 values are based on statistically significant differences.

Results

Cytochrome P450 1A induction measured with CEC

HepG2 and H4IIE cells were exposed for 24 h to a dose-range of 119 different compounds. Thereafter the CYP1A activity was measured with CEC. Exposure to 95 of these compounds did not affect CYP1A activity (Table 1). However, 24 compounds caused an induction of the CYP1A activity in either HepG2, H4IIE, or both cell lines. These compounds are listed in Table 2 that shows also the EC50 values and induction factors (ratio of treated cells:0.1% DMSO control). Dose response curves are presented in Figure 1.

The results clearly show that there was a difference between CYP1A induction in the human HepG2 and rat H4IIE cell line. More compounds caused CYP1A induction in H4IIE cells and most EC50 values were lower than in HepG2 cells. TCDD was as expected the most potent CYP1A inducer in both H4IIE and HepG2 cells. The EC50

value was $1.35x10^{-9}$ M in H4IIE cells and 36-times higher in HepG2 cells. Furthermore, benzo[a]pyrene (B[a]P), β-naphthoflavone (BNF), dihydroergotamine mesylate (DHE), 2,4-dinitrophenol, ellipticin, flutamide, indigo, indirubin, 3-methylcholanthrene (3MC), Org C, Org D, PCB 77, PCB 105, PCB 114, PCB 118, PCB 123, PCB 126, PCB 156, PCB 157, and PCB 167 induced the CYP1A activity in the H4IIE cell line. Of these 20 compounds B[a]P, BNF, indigo, indirubin, 3MC, Org C, PCB 77, PCB 114, PCB 123, PCB 126, and PCB 167 also induced the CYP1A activity in HepG2 cells. Menadione, Org A, and Org B were HepG2 specific CYP1A inducers. Moreover, Org C was more potent in HepG2 than in H4IIE cells.

Cytochrome P450 1A1 induction measured with Luciferin-CEE

Luc-CEE was used to measure the CYP1A1 induction in HepG2 and H4IIE cells. The same compounds except the four Org compounds were tested. Results were comparable with the results measured with CEC. The same compounds with exception of 2,4-dinitrophenol caused CYP1A1 induction in either HepG2, H4IIE, or both cell lines (Table 2). Although not statistically significant, 2,4-dinitrophenol showed a tendency to induction (1.8-fold) in H4IIE cells. The EC50 values of the CEC and Luc-CEE assay were almost similar. Nevertheless, the induction factors were for most compounds higher in the Luc-CEE assay.

Table 1. Compounds that do not activate CYP1A activity in the human HepG2 and rat H4IIE cell line

Acetylsalicylic acid	Ferrous sulfate	Orphenadrine citrate
α-Naphthoflavone	Fluorouracil	Papaverine
Quinoline	Furafylline	PCB 169
Aminophylline	Gentamicin A	PCB 189
Amiodarone HCl	Hexachlorobutadiene	Paracetamol
Antazoline mesylate	2,5-Hexanedione	Perhexiline
Atropine sulfate	Hydralazine HCl	Perphenazine
Bishydroxycoumarin	Hydrochlorothiazide	Phenobarbital sodium
Bromobenzene	Hydroxychloroquinone sulfate	Phentolamine mesylate
Carbon tetrachloride	4-Hydroxytamoxifen	Quinidine
Chlorpromazine HCl	ICI 164.384 (anti-estrogen)	Quinidine sulfate
Cisplatin	Imipramine HCl	Raloxifen
CITCO	Indomethacin	Reserpine
Clozapine	Iodoacetate	Rifampicin
Colchicine	Iproniazid HCl	Rotenone
Cyclophosphamide	Isoprenaline HCl	RU 1881
Cytarabine	Ketoconazole	RU 58668
Dacarbazine	Labetalol	Salicylamide
Dantrolene sodium	L-DOPA	Strychnine nitrate
Dehydroepiandrosterone	Levonorgestrel	Sulfamoxole
Dexamethasone	Medroxyprogesterone acetate	Sulphaphenazole

Table 1. Continued

Diclofenac	Melphalan	Tacrine
Diethyldithio-carbamic acid	Methadone HCl	Tamoxifen
Diethylstilbestrol	Methampyrone	Tertiair-butyl-hydroperoxide
Digoxin	Methotrexate	Testosterone
2,7-Dinitrofluorene	7α-Methyltestosterone	Tetracycline HCl
Dopamine HCl	17α-Methyltestosterone	Tolcapone
Doxorubicin	Naphazoline nitrate	Tularik 901317
Erythromycin	N-ethylmaleimide	Uramustine
Estradiol-17β	Nitrofurantoin	
Ethacrinic acid	Nitropyrene	
Ethinylestradiol-17β	4-Nitrosoquinoline-1-oxide	
Ethionine	Noscapine HCl	

Table 2. Cytochrome P450 1A induction measured with CEC and Luc-CEE in human HepG2 and rat H4IIE cells. Compounds indicated in bold and italic show rat and human specific CYP1A activation, respectively. The EC50 values and induction factors (IF) are shown.

Compound	**HepG2**				**H4IIE**			
	CEC		Luc-CEE		CEC		Luc-CEE	
	EC50 (mol/l)	IF	EC50 (mol/l)	IF	EC50 (mol/l)	IF	EC50 (mol/l)	IF
B[a]P	$3.73x10^{-6}$	10.2	$2.00x10^{-6}$	2.8	$2.08x10^{-8}$	16.7	$8.00x10^{-8}$	33.2
BNF	$1.22x10^{-5}$	20.3	$3.16x10^{-6}$	9.2	$2.95x10^{-8}$	24.6	$3.16x10^{-8}$	52.9
DHE	$1.21x10^{-5}$	7.9	$6.09x10^{-6}$	11	$3.35x10^{-6}$	2.43	$1.00x10^{-5}$	6.5
Dinitrophenol	$>1.00x10^{-4}$		$>1.00x10^{-4}$		$2.59x10^{-6}$	3.79	$>1.00x10^{-4}$	
Ellipticin	$>1.00x10^{-4}$		$>1.00x10^{-4}$		$1.00x10^{-8}$	4.6	$1.00x10^{-8}$	5.8
Flutamide	$>1.00x10^{-4}$		$>1.00x10^{-4}$		$7.89x10^{-7}$	13.1	$5.00x10^{-7}$	25.7
Indigo	$5.79x10^{-6}$	49.7	$3.85x10^{-6}$	116	$8.52x10^{-9}$	25.3	$3.16x10^{-8}$	63.0
Indirubin	$1.73x10^{-7}$	93	$1.27x10^{-7}$	368	$4.91x10^{-8}$	29	$2.00x10^{-7}$	68.3
Menadione	$1.29x10^{-5}$	16.9	$1.00x10^{-5}$	20.4	$>1.00x10^{-4}$		$>1.00x10^{-4}$	
3MC	$6.48x10^{-7}$	62.3	$5.22x10^{-7}$	222	$4.45x10^{-8}$	24.4	$3.16x10^{-8}$	69.5
Org A	$2.00x10^{-6}$	9.94	ND		$>1.00x10^{-4}$		ND	
Org B	$2.00x10^{-6}$	14.0	ND		$>1.00x10^{-4}$		ND	
Org C	$2.00x10^{-6}$	22.6	ND		$3.16x10^{-5}$	2.34	ND	
Org D	$>1.00x10^{-4}$		ND		$2.00x10^{-7}$	9.50	ND	
PCB 77	$8.27x10^{-6}$	7.9	$2.56x10^{-6}$	25.5	$4.35x10^{-8}$	17.5	$7.00x10^{-8}$	25.5
PCB 105	$>1.00x10^{-4}$		$>1.00x10^{-4}$		$>1.00x10^{-4}$	11.5	$>1.00x10^{-4}$	9.6
PCB 114	$>1.00x10^{-4}$	32.4	$>1.00x10^{-4}$	29.8	$1.30x10^{-6}$	13.6	$2.35x10^{-6}$	15.6
PCB 118	$>1.00x10^{-4}$		$>1.00x10^{-4}$		$>1.00x10^{-4}$	8.9	$>1.00x10^{-4}$	13.6
PCB 123	$>1.00x10^{-4}$	2.2	$>1.00x10^{-4}$	3.5	$>1.00x10^{-4}$	2.4	$>1.00x10^{-4}$	3.5
PCB 126	$1.65x10^{-6}$	50.6	$2.55x10^{-6}$	65.4	$1.00x10^{-9}$	21.6	$2.00x10^{-9}$	23.6
PCB 156	$>1.00x10^{-4}$		$>1.00x10^{-4}$		$5.37x10^{-6}$	16.0	$3.09x10^{-6}$	24
PCB 157	$>1.00x10^{-4}$		$>1.00x10^{-4}$		$8.15x10^{-6}$	19.7	$7.56x10^{-6}$	40.6
PCB 167	$>1.00x10^{-4}$	52.6	$>1.00x10^{-4}$	45.5	$3.55x10^{-8}$	15.3	$3.02x10^{-8}$	30.5
TCDD	$4.88x10^{-8}$	61.8	$4.00x10^{-8}$	250	$1.35x10^{-9}$	22.3	$1.25x10^{-9}$	78.5

Abbreviations: B[a]P=benzo[a]pyrene; BNF=β-naphthoflavone; DHE=dehydroergotamine mesylate; 3MC=3-methylcholanthrene; PCB=polychlorobiphenyl; TCDD=2,3,7,8-tetrachlorodibenzo-p-dioxin

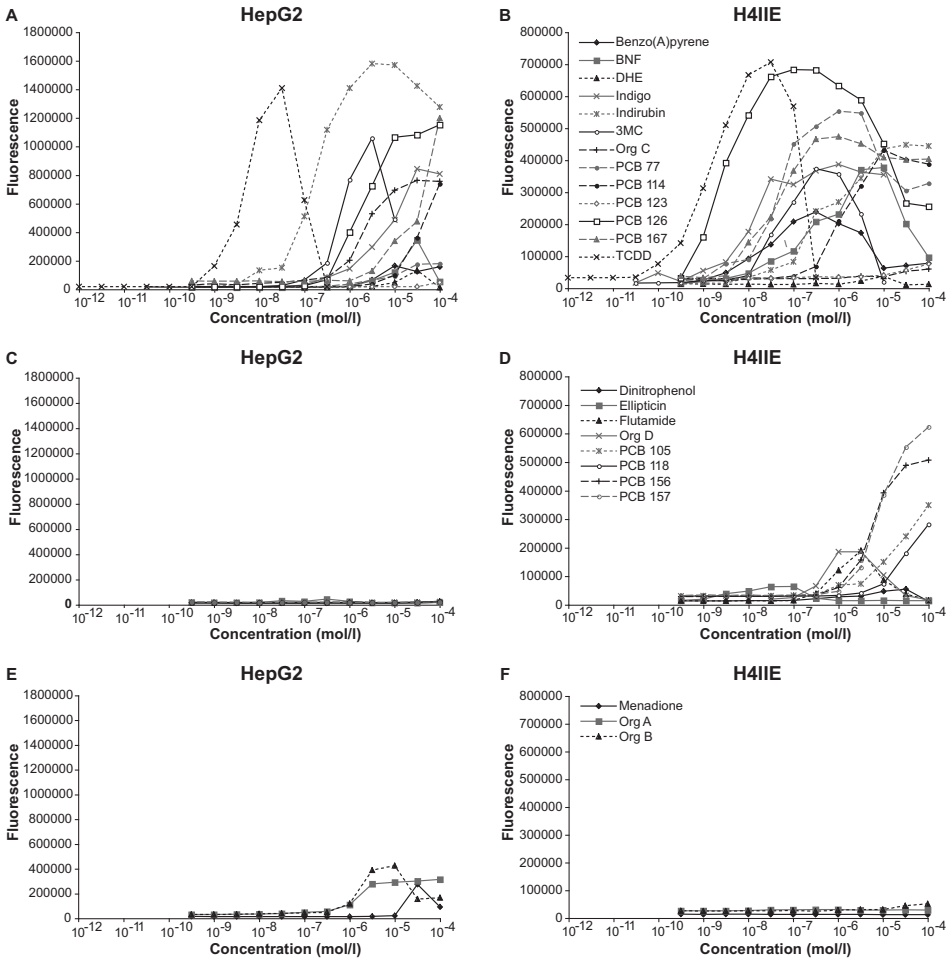

Figure 1. Induction of CYP1A activity measured with CEC in human HepG2 and rat H4IIE cells after 24 h exposure to the compounds. Of the 119 compounds, 13 induced CYP1A activity in both HepG2 and H4IIE cells (A and B), 8 showed H4IIE specific induction (C and D), and 3 compounds showed HepG2 specific induction (E and F). Results are presented as the mean of three independent measurements. The SD is not shown as it interferes with the reading of the marker spots. However, SD is <5% for all data points. Abbreviations: BNF=β-naphthoflavone; DHE=dehydroergotamine mesylate; 3MC=3-methylcholanthrene; TCDD=2,3,7,8-tetrachlorodibenzo-p-dioxin.

Cytochrome P450 1A1 and 1A2 competition

CYP1A1 and 1A2 competition in human and rat supersomes was measured for 115 compound, four compounds were skipped from analysis i.e. Org A, B, C, and D. In Figure 2 the dose-related competition for human and rat CYP1A1 and 1A2 is demonstrated for

a representative set of 8 compounds, i.e. B[a]P, ellipticine, furafylline, indigo, indirubin, ketoconazole, nitrofurantoin and 4-NQO. The efficacies (EFF) and IC50 values are shown in Table 3. Differences were observed between CYP1A1 and 1A2 competition in human and rat supersomes. Of the 115 tested compounds, 36 compounds inhibited human CYP1A1 activity for 50% or more. Human competition for CYP1A1 was found specific for 12 compounds, being β-naphthoflavone, cisplatin, dehydroergotamine mesylate, dopamine HCl, 17β-estradiol, 4-hydroxytamoxifen, noscapine HCl, papaverine, quinidine, quinidine sulfate, reserpine and RU 58668. The remaining 24 compounds also inhibited rat CYP1A1. Furthermore, rat specific competition for CYP1A1 was found for ten compounds.

Similar differences were observed for CYP1A2. Competition was found for 37 compounds of which 14 and 3 compounds showed specific competition for human and rat CYP1A2, respectively.

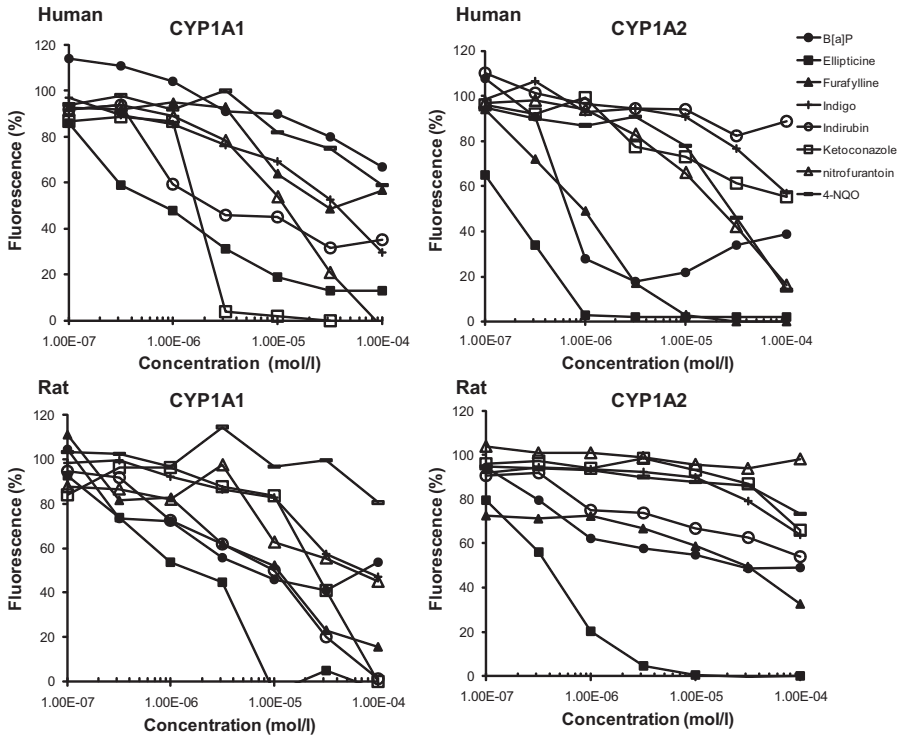

Figure 2. CYP1A1 and 1A2 competition in human and rat supersomes by benzo[a]pyrene (B[a]P), ellipticine, furafylline, indigo, indirubin, ketoconazole, nitrofurantoin and 4-nitrosoquinolineoxide-1-oxide (4-NQO). Results are presented as the mean of three independent measurements (0.1% DMSO control = 100%). The SD is not shown as it interferes with the reading of the marker spots. However, SD is <5% for all data points.

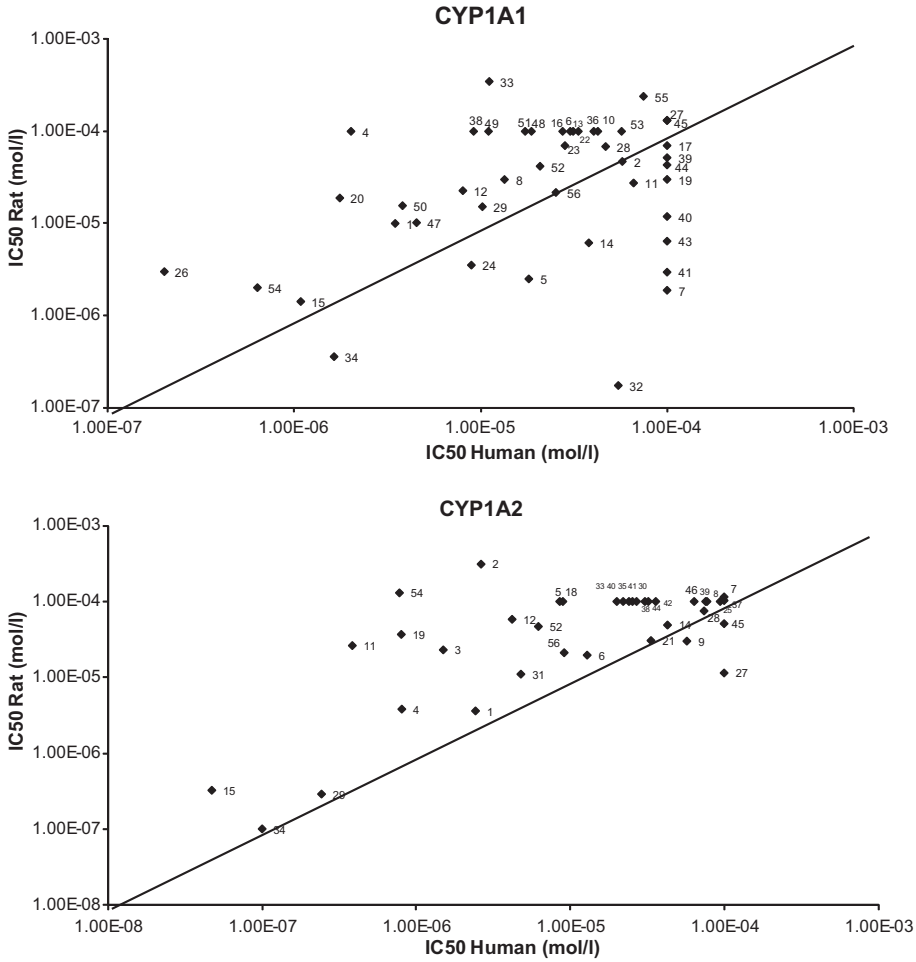

Figure 3. Comparison of the IC50 values of human versus rat for CYP1A1 and 1A2 competition. The numbers represent the compounds in Table 3. The solid line is the line of identity (x=y).

A x:y plot for the IC50 values of human versus rat competition for both CYP1A1 and 1A2 is shown in Figure 3. The numbers in the Figure 3 represent the compounds in Table 3. The solid line in Figure 3 is the line of identity (x = y). The outlier hydralazine HCl is not shown in the CYP1A1 plot. As can be seen in Figure 3, there are besides the human and rat specific CYP1A1 competitors, compounds that are almost equally potent in both species (compounds around the line of identity). However, the potency is for most compounds higher in human supersomes as compared to rat supersomes (compounds above the line of identity). The same is true for CYP1A2 as almost all competitors have a lower IC50 value in human supersomes as compared to rat supersomes.

Table 3. Compounds that show CYP1A1 and 1A2 competition in human and rat supersomes. The efficacy (EFF) and IC50 values are shown. Values are marked in bold when EFF>50% ($p<0.05$).

No	Compound	Human				Rat			
		CYP 1A1 inhibition		CYP1A2 inhibition		CYP1A1 inhibition		CYP1A2 inhibition	
		EFF (%)	IC50 (M)	EFF (%)	IC50 (M)	EFF (%)	IC50 (M)	EFF (%)	IC50 (M)
1	α-Naphthoflavone	**111**	**3.47x10^{-6}**	**104**	**2.43x10^{-6}**	**101**	**1.00x10^{-5}**	**98**	**3.60x10^{-6}**
2	Quinoline	**67**	**5.75x10^{-5}**	**95**	**2.64x10^{-6}**	**53**	**4.71x10^{-5}**	**56**	**3.11x10^{-4}**
3	B[a]P	33	>1.00x10^{-4}	**61**	**1.50x10^{-6}**	23	>1.00x10^{-4}	**51**	**2.30x10^{-5}**
4	BNF	**91**	**2.01x10^{-6}**	**83**	**8.07x10^{-7}**	11	>1.00x10^{-4}	**108**	**3.80x10^{-6}**
5	Chlorpromazine	**74**	**1.81x10^{-5}**	**72**	**8.56x10^{-6}**	**98**	**2.50x10^{-6}**	31	>1.00x10^{-4}
6	Cisplatin	**72**	**3.02x10^{-5}**	**82**	**1.29x10^{-5}**	43	>1.00x10^{-4}	**93**	**1.96x10^{-5}**
7	Clozapine	-3	>1.00x10^{-4}	**52**	**1.00x10^{-4}**	**107**	**1.88x10^{-6}**	**47**	**1.15x10^{-4}**
8	Dantrolene sodium	**91**	**1.34x10^{-5}**	**60**	**7.74x10^{-5}**	**75**	**3.00x10^{-5}**	39	>1.00x10^{-4}
9	DETC	37	>1.00x10^{-4}	**71**	**5.73x10^{-5}**	12	>1.00x10^{-4}	**87**	**3.00x10^{-5}**
10	DHE	**66**	**4.25x10^{-5}**	-6	>1.00x10^{-4}	19	>1.00x10^{-4}	-29	>1.00x10^{-4}
11	2,7-Dinitrofluorene	**64**	**6.61x10^{-5}**	**86**	**3.85x10^{-7}**	**53**	**2.75x10^{-5}**	**61**	**2.62x10^{-5}**
12	DNP	**69**	**8.01x10^{-6}**	**81**	**4.20x10^{-6}**	**49**	**2.27x10^{-5}**	**51**	**5.82x10^{-5}**
13	Dopamine HCl	**84**	**3.13x10^{-5}**	8	>1.00x10^{-4}	27	>1.00x10^{-4}	2	>1.00x10^{-4}
14	Doxorubicin	**73**	**3.80x10^{-5}**	**67**	**4.29x10^{-5}**	**90**	**6.16x10^{-6}**	**69**	**4.89x10^{-5}**
15	Ellipticine	**87**	**1.08x10^{-6}**	**98**	**4.71x10^{-8}**	**104**	**1.42x10^{-6}**	**100**	**3.23x10^{-7}**
16	Estradiol-17β	**73**	**2.75x10^{-5}**	30	>1.00x10^{-4}	4	>1.00x10^{-4}	-2	>1.00x10^{-4}
17	Ethacrinic acid	14	>1.00x10^{-4}	20	>1.00x10^{-4}	**53**	**7.01x10^{-5}**	9	>1.00x10^{-4}
18	Flutamide	30	>1.00x10^{-4}	**71**	**8.97x10^{-6}**	-16	>1.00x10^{-4}	9	>1.00x10^{-4}
19	Furafylline	43	>1.00x10^{-4}	**100**	**8.01x10^{-7}**	**77**	**3.01x10^{-5}**	**56**	**3.68x10^{-5}**
20	HCBD	**83**	**1.75x10^{-6}**	5	>1.00x10^{-4}	**59**	**1.89x10^{-5}**	5	>1.00x10^{-4}
21	Hydralazine HCl	**69**	**5.99x10^{-5}**	**58**	**3.35x10^{-5}**	**66**	**5.80x10^{-8}**	**55**	**3.05x10^{-5}**
22	4-OH-Tamoxifen	**81**	**3.34 x10^{-5}**	-14	>1.00x10^{-4}	6	>1.00x10^{-4}	17	>1.00x10^{-4}
23	Indigo	**71**	**2.83x10^{-5}**	43	>1.00x10^{-4}	**53**	**7.00x10^{-5}**	36	>1.00x10^{-4}
24	Indirubin	**65**	**8.90x10^{-6}**	11	>1.00x10^{-4}	**99**	**3.53x10^{-6}**	46	>1.00x10^{-4}
25	Isoprenaline HCl	10	>1.00x10^{-4}	**64**	**9.43x10^{-5}**	7	>1.00x10^{-4}	14	>1.00x10^{-4}
26	Ketoconazole	**100**	**2.00x10^{-7}**	45	>1.00x10^{-4}	**100**	**3.00x10^{-6}**	-14	>1.00x10^{-4}
27	L-DOPA	18	>1.00x10^{-4}	-62	>1.00x10^{-4}	**56**	**1.31x10^{-4}**	**112**	**1.14x10^{-5}**
28	Melphalan	**66**	**4.68x10^{-5}**	**53**	**7.41x10^{-5}**	**63**	**6.84x10^{-5}**	**59**	**7.51x10^{-5}**
29	Menadione	**85**	**1.02x10^{-5}**	**102**	**2.43x10^{-7}**	**80**	**1.52x10^{-5}**	**93**	**2.88x10^{-7}**
30	Methotrexate	18	>1.00x10^{-4}	**88**	**2.70x10^{-5}**	4	>1.00x10^{-4}	28	>1.00x10^{-4}
31	3MC	25	>1.00x10^{-4}	**74**	**4.79x10^{-6}**	8	>1.00x10^{-4}	**57**	**1.10x10^{-5}**
32	7α-methyl-testosterone	**64**	**5.47x10^{-5}**	6	>1.00x10^{-4}	**116**	**1.74 x10^{-7}**	10	>1.00x10^{-4}
33	Nitrofurantoin	**103**	**1.11x10^{-5}**	**84**	**2.01x10^{-5}**	**55**	**3.48x10^{-4}**	2	>1.00x10^{-4}
34	Nitropyrene	**83**	**1.63x10^{-6}**	**92**	**1.00x10^{-7}**	**99**	**3.58x10^{-7}**	**108**	**1.00x10^{-7}**
35	4-NQO	41	>1.00x10^{-4}	**86**	**2.41x10^{-5}**	19	>1.00x10^{-4}	27	>1.00x10^{-4}
36	Noscapine HCl	**65**	**4.04x10^{-5}**	1	>1.00x10^{-4}	12	>1.00x10^{-4}	5	>1.00x10^{-4}
37	Orphenadrine citrate	33	>1.00x10^{-4}	40	>1.00x10^{-4}	15	>1.00x10^{-4}	**53**	**1.03 x10^{-4}**

Table 3. Continued

No	Compound	Human				Rat			
		CYP1A1 inhibition		CYP1A2 inhibition		CYP1A1 inhibition		CYP1A2 inhibition	
		EFF (%)	IC50 (M)	EFF (%)	IC50 (M)	EFF (%)	IC50 (M)	EFF (%)	IC50 (M)
38	Papaverine	**92**	**$9.15x10^{-6}$**	**71**	**$3.06x10^{-5}$**	38	$>1.00x10^{-4}$	18	$>1.00x10^{-4}$
39	PCB 105	11	$>1.00x10^{-4}$	**42**	**$7.59x10^{-5}$**	**57**	**$5.18x10^{-5}$**	22	$>1.00x10^{-4}$
40	PCB 114	-8	$>1.00x10^{-4}$	**69**	**$2.21x10^{-5}$**	**95**	**$1.19x10^{-5}$**	34	$>1.00x10^{-4}$
41	PCB 118	22	$>1.00x10^{-4}$	**54**	**$2.54x10^{-5}$**	**86**	**$2.96x10^{-6}$**	24	$>1.00x10^{-4}$
44	PCB 167	28	$>1.00x10^{-4}$	**53**	**$3.22x10^{-5}$**	**72**	**$4.32x10^{-5}$**	45	$>1.00x10^{-4}$
47	Perphenazine	**101**	**$4.52x10^{-6}$**	1	$>1.00x10^{-4}$	**82**	**$1.02x10^{-5}$**	1	$>1.00x10^{-4}$
48	Quinidine	**76**	**$1.87x10^{-5}$**	-2	$>1.00x10^{-4}$	27	$>1.00x10^{-4}$	30	$>1.00x10^{-4}$
49	Quinidine sulfate	**89**	**$1.10x10^{-5}$**	24	$>1.00x10^{-4}$	20	$>1.00x10^{-4}$	5	$>1.00x10^{-4}$
50	Raloxifen	**100**	**$3.80x10^{-6}$**	43	$>1.00x10^{-4}$	**80**	**$1.56x10^{-5}$**	23	$>1.00x10^{-4}$
51	Reserpine	**59**	**$1.73x10^{-5}$**	47	$>1.00x10^{-4}$	23	$>1.00x10^{-4}$	-5	$>1.00x10^{-4}$
52	Rifampicin	**74**	**$2.08x10^{-5}$**	**90**	**$6.22x10^{-6}$**	**60**	**$4.18x10^{-5}$**	**58**	**$4.68x10^{-5}$**
53	RU 58668	**102**	**$5.70x10^{-5}$**	2	$>1.00x10^{-4}$	-15	$>1.00x10^{-4}$	39	$>1.00x10^{-4}$
54	Tacrine	**115**	**$6.32x10^{-7}$**	**108**	**$7.79x10^{-7}$**	**131**	**$2.01x10^{-6}$**	**50**	**1.30E-04**
55	Testosterone	**64**	**$7.47x10^{-5}$**	-6	$>1.00x10^{-4}$	**45**	**$2.40x10^{-4}$**	-4	$>1.00x10^{-4}$
56	Tolcapone	**91**	**$2.53x10^{-5}$**	**92**	**$9.15 x10^{-6}$**	**110**	**$2.17x10^{-5}$**	**92**	**$2.11x10^{-5}$**

Abbreviations: B[a]P=benzo[a]pyrene; BNF=β-naphthoflavone; DETC=Diethyldithio-carbamic acid; DNP=2,4 dinitrophenol; 3MC=3-methylcholanthrene; 4-NQO=4-nitrosoquinolineoxide-1-oxide

Discussion

The present study focused on CYP1A induction, (non-)competitive inhibition and species differences between human and rat. Strong AhR activation leads to a series of toxic effects and consequently it may be useful for the pharmaceutical industry to screen for and deselect NCEs that are strong CYP1A inducers (AhR activators) in the early phase of drug development.

Primary cultures of hepatocytes can be used for CYP1A induction studies, however, donor variation and a high number of compounds in the early developmental phase make the use of cell lines preferable. Two such cell lines may be the human and rat hepatoma cell lines HepG2 and H4IIE. Previously we demonstrated that the effects of AhR agonists in HepG2 on cytochrome P450 enzymes are similar to the effects observed in primary human hepatocytes [27]. In line with this it was reported that HepG2 cells are a better model reflecting CYP1A induction in human hepatocytes than hepatocytes from Rhesus monkeys and Sprague-Dawley rats [28]. Others found that the H4IIE cell line is a good model to study CYP1A induction reflecting effects in primary rat hepatocytes [26]. Thus it seems that HepG2 and H4IIE cells are good cell lines to study human and

rat CYP1A induction, respectively.

For induction studies the fluorogenic substrate CEC was used. Fluorescence detection of the deethylation of CEC was reported to be 50- up to 100-times more sensitive than that of ethoxyresorufin, primarily because of the faster turnover rate of CEC [29]. CEC is not specific for a CYP1A isoform, it reacts with both human and rat CYP1A1 and 1A2 with a preference for CYP1A1 [30]. Besides CEC, the luminogenic substrate Luc-CEE was used for measuring CYP1A induction. The results were quite similar to results observed with CEC. An advance of this substrate may be it's specificity for human and rat CYP1A1 (Promega). There is also a CYP1A2 specific luminogenic substrate (Luciferin-BE) available. In the present study this substrate was not used because it reacts only with human CYP1A2 and shows no reactivity for rat CYP1A2 (data not shown).

Of the 119 compounds that were tested, 24 compounds were able to induce the CYP1A activity in either human HepG2, rat H4IIE, or both cell lines. Pronounced differences were observed between induction in the cells of human and rat origin. Zeiger et al. [26] reported similar differences. In their study HepG2 and H4IIE cells were also exposed to the dioxin like PCBs 77, 105, 114, 118, 123, 126, 156, 157, 167, 169, and 189. In H4IIE cells, they found in concordance with the present study induction after exposure to PCB 77, 105, 114, 118, 123, 126, 156, 157, and no induction after exposure to PCB 189. However, in contradiction we did not find induction with PCB 169 in H4IIE cells. Induction in HepG2 cells was found for less compounds at higher doses. In both studies induction was measured after exposure to PCB 77, 114, and 126. In contradiction with Zeiger et al. [26], we found induction in HepG2 cells by PCB 123 and 167. The results suggest that the rat cell line H4IIE is more sensitive to detect CYP1A inducers than the HepG2 cell line.

The high sensitivity of rat for CYP1A inducers was also reported by Silkworth et al. [28] , who found CYP1A induction at much lower concentrations after exposure to PCB 126 and Aroclor 1254 in rat than human cells. A study by Aluru et al. [31] also revealed species differences. While in humans α-naphthoflavone (ANF) is an AhR antagonist, in rainbow trout hepatocytes it was a partial agonist.

Although mono-ortho PCBs such as PCB 114, PCB126, and PCB167 are considered as weak AhR agonists [32] they showed a high potency in the present study. Peters et al. [32] showed that after purification of mono-ortho PCBs with active charcoal, mono-ortho PCBs showed only a low potency in an AhR-EGFP reporter assay mouse Hepa1c1c7 and rat H4IIE cells. The purity of the PCBs in the present study was 99%. Therefore we do not expect large effects of impurities on CYP1A induction, however we cannot exclude that contaminations with AhR agonists of high activity had an effect on the activity.

The importance of deselecting CYP1A inducers was stressed by Org D. This rat specific CYP1A inducer caused non-genotoxic carcinogenicity in in vivo rat studies

(unpublished in house data).

We observed that Org A and B were HepG2 specific CYP1A inducers and Org C was much more potent in the HepG2 cell line. Menadione was also active in HepG2 cells and not in H4IIE cells. Apparently there are CYP1A inducers that are more potent in humans than rats. In line with this, in human, but not in mouse and in rat, the anti-ulcer drug omeprazole has been reported to induce CYP1A2 [33-35]. Overall these observations make screening in cell lines from both rat and human a useful strategy.

Recently Sonneveld et al. [36] showed that glucocorticoids enhance the induction of CYP1A1 and other AhR target genes in rat H4IIE cells but not in human cells. In the present study we did not observe an effect of dexamethasone in H4IIE cells also not when charcoal treated serum was used (data not shown).

There are also other methods available for screening CYP1A inducers and/or AhR activators. Two often used methods are the CALUX bioassay [37] and the use of 101L cells [38]. In the CALUX bioassay rat H4IIE and mouse Hepa1c1c7 cells containing a luciferase gene under control of dioxin responsive elements are used. A disadvantage of this assay is that rodent cells are used which not always reflect effects in humans. The 101L cell line is a stable cell line derived from HepG2 cells which contain a human CYP1A1 luciferase reporter. An advantage of this system may be that substrate inhibition of CYP1A1 does not play a role. However, in our assay we included a washing step after compound incubation which reduced substrate inhibition of CEC.

Ellipticine showed CYP1A induction in the H4IIE cell line. In HepG2 cells induction was just below 2-fold and directly after the increase in activity the signal dropped sharply again. The problem with the detection of ellipticine in the HepG2 cell line was cytotoxicity. At the concentration where this compound starts to activate the AhR it shows also a strong cytotoxic effect [39, 40]. All other compounds in the present study were tested for cytotoxicity by using the glutathione depletion and calcein-AM assay [39, 40]. These results revealed (data not shown) that compounds not showing CYP1A induction in HepG2 cells but showing CYP1A induction in H4IIE cells were not missed in HepG2 cells because of cytotoxicity.

CYP1A induction can easily be used in the early developmental phase to detect compounds that may show dioxin-like toxicity. However, care should be taken to deselect such compounds directly as CYP1A activation does not necessarily mean dioxin-like toxicity. There are for example marketed therapeutics like omeprazole, leflunomide, flutamide, and nimodipine which are safely used but have been proven to be AhR agonists [41]. Furthermore AhR agonists like indirubin and meisoindigo have been shown to be effective in the treatment of chronic myelogenous leukemia [42]. Hu et al. [41] also demonstrated that induction of CYP1A1 is a non-specific marker of direct AhR affinity. In the present study we measured CYP1A induction and not AhR activation. Therefore also compounds are detected that alter the CYP1A expression by pathways in addition

to those mediated by the AhR. Induction of CYP1A has been reported after oxidative stress [43, 44]. These compounds do not have the side-effects reported for some AhR activators, however, an adverse side-effect of these compounds may be the effect of increased CYP1A activity on the efficacy of the compound itself or the induction could result in drug-drug interactions.

The potentially toxic effect of CYP1A inducers can be confirmed by using in vitro or in vivo transcriptional profiling. By studying the change of a broad set of genes dioxin-like toxicity may be predicted more precisely. Consequently a compound may be deselected or selected.

Thus summarized, CYP1A induction can be used as a pre-screening tool to detect compounds that may show dioxin-like toxicity. However, further studies are needed to confirm this dioxin-like toxicity. When during drug development equally potent compounds without CYP1A induction are available, selection of these compounds may be preferred to avoid possible safety problems.

Besides species differences between CYP1A1 induction, species differences between the AhR receptor of human and rat could lead to differentially regulated gene expression. Recently it has been shown by Flaveny et al. [45] that differences between the transactivation domains of the human and mouse AhR result in differential recruitment of co-activators. It is likely that this leads to a divergent regulation of target genes. Differences in the recruitment of co-activators between the human and rat AhR receptor have not been studied yet, however, similar differences as found between human and mouse may exist.

CYP1A1 and 1A2 competition assays were performed by using human and rat supersomes. The same set of compounds with exception of four in-house NCEs was tested. Like for induction species differences were observed. Of the 115 compounds 46 showed CYP1A1 competition. Competition was human and rat specific for 12 and 10 compounds, respectively. CYP1A2 competition was observed for 37 compounds of which 14 and 3 compounds showed human and rat specific inhibition, respectively. The similarity between the amino acid sequence of human and rat CYP1A1 is 79%, and of human and rat CYP1A2 73%. The difference in amino acid sequences might account for the differences in competition. Other studies also reported species differences between CYP1A metabolism in humans and rats. Shinkyo et al. [46] studied the metabolism of TCDD and other polychlorinated dibenzo-p-dioxins in human and rat microsomes and found significant species differences. Boogaards et al. [47] compared the CYP activities towards marker CYP substrates for human, rat, rabbit, dog, and micropig microsomes. They found that in none of the tested species metabolism was similar to CYP metabolism in man. With respect to CYP1A human metabolism was most similar to mouse, followed by rabbit, micropig, rat and dog.

In summary, we used a medium-/high-throughput assay in a 96-well format for detecting

CYP1A inducers in the human HepG2 and rat H4IIE cell line. Moreover, CYP1A1 and 1A2 competition assays were performed by using human and rat supersomes in a 384-well high-throughput assay. The induction and competition assays revealed for several compounds a species difference between human and rat. Therefore, parallel screening in both species may be a useful strategy for toxicity screening in the early discovery phase of drug development.

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Chapter 9

Summary, discussion, conclusion and additional considerations

Background

In drug development the costs spend on R&D have increased tremendously during the last decades. In spite of the increase in R&D expenses, the development of new assay methods, new techniques in liquid handling, robotics, analytical tools and software, the number of approved new drugs on a yearly basis has declined.

In drug development, toxicity is an important factor for attrition, resulting in a failure rate of 30%-40%. Hepatotoxicity, nephrotoxicity, cardiovascular safety, reproductive toxicity, developmental toxicity (teratogenicity), genotoxicity and carcinogenicity are important causes for attrition in safety assessment.

Screening on these aspects of toxicity in the early discovery phase of drug development and using these data for compound optimization and deselection might proof to be a strategy that results in drug development candidates with an improved success rate. The present thesis is focused on early screening for genotoxicity and carcinogenicity.

In regulatory genotoxicity testing a stepwise approach (tiered approach) is applied and in this strategy in vitro assays are used as a first step to see whether the test compounds have intrinsic genotoxic activity. These tests, especially when positive, are followed by in vivo tests that are designed to assess the relevance of the in vitro result for the in vivo situation.

The regulatory test strategy consists of a battery of tests to assess gene mutations and chromosome damage (clastogenicity and aneugenicity). The standard test battery required for genotoxicity testing consists of (1) the Ames assay to detect gene mutations in bacteria and (2) an in vitro chromosome aberration (CA) + (or) mouse lymphoma TK assay (MLA) in mammalian cells to detect chromosome damage. A combination of these in vitro screens has a high sensitivity for genotoxic potential (Table 1). These tests are then followed by (3) an in vivo chromosome damage assay (chromosome aberration or micronucleus assay). Depending on the results, additional testing might be necessary. For the determination of carcinogenic potential, the induction of tumors is monitored in a 2-year, life time exposure of mice and rats. The regulatory tests to detect genotoxic potential are performed in the preclinical phase before the first into man studies are initiated. Testing for carcinogenic potential is performed later on during the clinical development.

The regulatory tests to detect genotoxic and carcinogenic potential have in general a low-throughput, need a high amount of compound, and are laborious. Therefore these assays are at least in their current format not suitable for medium- or high-throughput screening in the early discovery phase.

In recent years some progress has been made with earlier screening procedures for determining genotoxic and carcinogenic potential at the end of the discovery phase.

However, generally these assays are performed at a relatively late time point in the drug discovery phase at which chemical optimization of compounds on both pharmacological activity as well as on adverse properties is hard to achieve. Only a few genotoxicity and carcinogenicity assays for such a strategy of early phase testing are available and the throughput of these assays is in general low and mostly these assays were not thoroughly validated. An additional drawback is that the current in vitro assays for the detection of genotoxicity give a high rate of false positive results (low specificity see Table 1), which makes application in the early discovery phase of drug development cumbersome.

The goal of this thesis was therefore to develop improved in vitro assays for detecting the genotoxic and non-genotoxic carcinogenic potential of chemicals, validate these assays with proper reference compounds and to develop a strategy for application of these assays in the early discovery phase of drug development. To this end assay systems based on bacteria, yeast, and rodent and human cell lines were developed and validated (Table 1). In the case of human cell lines, the focus was on the HepG2 cell line as the properties of HepG2 cells are expected to give a good prediction for in vivo genotoxicity because these cells display active p53, active DNA repair and have retained activities of phase I and II metabolizing enzymes.

The next sections summarize and discuss the results obtained in the present thesis. The first section summarizes, based on Chapter 2, the results obtained with the bacterial based reporter assay Vitotox™. This assay was evaluated as a rapid prescreen for the regulatory Ames test. Besides assays that are able to detect gene mutations (bacterial mutagenicity) a prescreen for genotoxicity in the early drug discovery phase should be able to rapidly detect compounds that cause chromosome damage (clastogenicity and aneugenicity). For this reason model systems in yeast and mammalian cells were evaluated. Section 2 summarizes, based on results in Chapter 2 and 7, the results obtained with the yeast based RAD54 reporter assay. The third section summarizes the results obtained with the human HepG2 cell line, which were described in Chapters 3, 4, 5, 6 and 7. The third section starts with summarizing the results from the characterization of cytochrome P450 and phase II metabolism in HepG2 cells (Chapters 3 and 4). Then the results from the development and evaluation of a high content screening in vitro micronucleus assay in the HepG2 cell line as well as in the commonly used Chinese hamster ovary cell line k1 (CHO-k1) are described (Chapter 5). The third part of the section summarizes, the results from a gene expression profiling study in HepG2 cells to find sensitive and specific biomarkers (gene subsets) for genotoxicity (Chapter 6). Based on this study luciferase based reporter assays with individual genes were developed and evaluated in the HepG2 cell line (Chapter 7). The third section is finalized with a summary of these results. Besides the detection of compounds with genotoxic potential it would be useful to be able to detect non-genotoxic carcinogens in the early discovery phase of drug development. Section four will focus on the detection of non-genotoxic carcinogenic potential caused

by aryl hydrocarbon receptor activators and species differences between rat and human. After summarizing the results from all the individual assays, combinations of the assays will be discussed for the development of an early screening strategy. Finally an overall conclusion is given, followed by additional considerations for the future.

The detection of genotoxicity with the bacterial based Vitotox™ test

A HTS alternative for the rapid detection of bacterial mutagenicity with the Ames assay, might be an assay, in which the induction of genes involved in repair of bacterial DNA damage (SOS-response) can be measured for the identification of the mutagenic potential of compounds. An assay that is based on this principle is the Vitotox™ assay, which is a bacterial reporter assay in *Salmonella typhimurium* based on activation of the SOS-response with a recN promoter controlled luminometric read-out. In Chapter 2 the Vitotox™ was evaluated as early screen for bacterial mutagenicity.

A recommended ECVAM compound list was used for the validation of the genotoxicity assays. This compound list consists of 20 well defined genotoxic carcinogens and 42 non-genotoxic compounds. Of the 20 genotoxic carcinogens 14 compounds are positive in the Ames test. The remaining genotoxins cause chromosome damage but give negative or equivocal results in the Ames assay. The 42 non-genotoxic compounds (non-carcinogens and non-genotoxic carcinogens) contain 19 compounds that give often false positive results in in vitro mammalian genotoxicity assays.

Moreover, an additional set of 192 compounds was used to broaden this validation study. The compounds of this additional set can be classified as non-genotoxins and genotoxins and consist of both in-house compounds from the legacy N.V. Organon and reference compounds. The compound list contains several steroidal compounds that have been reported as being clastogenic or aneugenic. Of these 192 compounds, Ames data were available for 145 compounds, mammalian in vitro genotoxicity data for 124 compounds and in vivo genotoxicity data for 70 compounds.

The sensitivity (percentage of genotoxic carcinogens that tested positive) of the Vitotox™ assay with respect to the ECVAM compound list was 70% (14/20). Excluding clastogenic and aneugenic compounds that gave negative or equivocal results in the Ames test, resulted in a sensitivity of 79% (11/14). The Vitotox™ assay gave a low number of false positive results as the specificity (percentage of non-genotoxic compounds that tested negative) was 93% (39/42).

Table 1. Overview of the regulatory in vitro genotoxicity tests and the high-throughput models that will be validated as prescreens for these tests.

Endpoint	Regulatory test(s)	Sensitivity	Specificity	Proposed high-throughput models
1. Bacterial mutagenicity (gene mutations)	Ames	58.5% (318/541)	73.9% (130/176)	Bacterial based Vitotox™ reporter assay (Chapter 2)
2. Mammalian genotoxicity (chromosome damage: clastogenicity and aneugenicity)	MLA + CA	81.3% (165/203)	27.1% (26/96)	Yeast based RadarScreen (Chapter 2) CHO-k1 HCS IVMN (Chapter 5) HepG2 HCS IVMN (Chapter 5) HepG2 luciferase based genotoxicity reporters (Chapter 7)
1 + 2	Ames + MLA + CA	84.7 % (171/202)	22.9% (22/96)	

Abbreviations: HCS=high content screening; IVMN=in vitro micronucleus assay; MLA=mouse lymphoma TK assay

The results with respect to the additional list of 192 compounds confirmed the results found with the ECVAM compound list. The Vitotox™ assay showed a high correlation with the Ames test of 91% (sensitivity, 86% (42/49); specificity, 94% (90/96)). The correlation with in vitro mammalian genotoxicity and in vivo mammalian genotoxicity results was much lower with respectively 50% (sensitivity 27% (21/78); specificity 89% (41/46)) and 54% (sensitivity 33% (13/38); specificity 78% (25/32)).

The overall results showed that the Vitotox™ assay is a good and rapid prescreen for bacterial mutagenicity. The regulatory Ames and Vitotox™ gave similar scores for the compounds that were tested. The throughput of the assay (16 compounds per day) and low amount of compound needed (5 mg) make the Vitotox™ more applicable in the early phase of drug discovery than the Ames assay or the miniaturized versions of the Ames assay. In comparison, even miniaturized versions of the Ames assay still need approximately 300 mg compound and have a throughput of less than 5 compounds per week [1]. As a consequence these assays are generally performed at a later time point in the drug discovery phase which renders compound optimization on both pharmacological properties as well as on genotoxicity more difficult. Complementary to the assessment of bacterial mutagenicity with the Vitotox™ assay the results in the Chapter 2 showed that for the rapid detection of chromosome damage additional testing is needed.

The detection of genotoxicity with the yeast based RadarScreen assay

Eukaryotic cell based systems are needed to detect compounds that cause chromosome damage (structural damage, clastogenicity; numerical damage, aneugenicity). In this aspect the eukaryotic yeast based RadarScreen was validated (Chapter 2**)**. The RadarScreen assay is a RAD54 promoter-linked β-galactosidase reporter assay of which the β-galactosidase expression can be quantified luminometrically. The level of luminescence might be a good measure for DNA damage as RAD54 plays a pivotal role in DNA repair in yeast.

The reproducibility of the assay was tested with the genotoxic reference compounds methyl methane sulphonate and benzo[a]pyrene and appeared to be good. Validation with respect to the ECVAM compound list resulted in a correlation with (in vivo) genotoxicity of 79% (sensitivity, 70% (14/20); specificity, 83% (35/42)). For the additional set of 192 compounds, the RadarScreen assay had a correlation with in vitro mammalian genotoxicity (clastogenicity) of 76% (sensitivity, 77% (59/77); specificity, 74% (34/46)).

Validation with the ECVAM compound list indicated that the number of falsely predicted

in vivo genotoxicity results is low, whereas results with the additional compound set showed the opposite with a predictivity of only 54% (sensitivity, 68% (25/37); specificity, 38% (12/32)). Especially the steroids in the second list of compounds gave false positive results for in vivo genotoxicity. The exact reason for these results obtained cannot be given. A lack of or (species) difference in metabolizing enzymes might have been the reason for the low predictivity. In addition, well known aneugens were difficult to detect with the RadarScreen assay.

Thus although a good predictivity for in vitro mammalian genotoxicity was measured, the predictivity for in vivo genotoxicity was low. Therefore other assays are needed.

The detection of genotoxicity with the human HepG2 cell line

A disadvantage of most proliferative carcinogenic cell lines is that they have lost their responsiveness for p53 activation, DNA repair response systems and/or their metabolizing capacities. Impairment of these systems might be the reason for the low in vitro predictivity of in vivo genotoxicity. The application of the human hepatoma cell line HepG2 might be useful in this aspect as HepG2 cells display active p53 protein, active DNA repair systems and have retained activities of phase I and II enzymes.

Several studies were performed with the HepG2 cell line, including the following investigations: (1) phase I and II metabolism was characterized (Chapters 3 and 4), (2) a high content screening in vitro micronucleus assay was developed and validated in HepG2 cells as well as in CHO-k1 cells, of which the latter are commonly used in regulatory in vitro micronucleus assays (Chapter 5), (3) transcriptomics in HepG2 cells exposed to genotoxic and non-genotoxic liver toxicants was used to assess biomarkers for genotoxicity (Chapter 6), (4) and finally, based on these transcriptomics data, luciferase based reporter assays for the detection of genotoxic potential were developed and validated (Chapter 7).

Characterization of phase I and II metabolism in the HepG2 cell line

Metabolism can result in toxification and detoxification of compounds. Knowledge of the metabolic status of cell lines used for in vitro genotoxicity testing is a prerequisite to understand the results obtained and to pinpoint at weaknesses and strengths of the in vitro genotoxicity assays being carried out. Therefore, a detailed metabolic characterization of the HepG2 cell line was performed, i.e. messenger RNA (mRNA) levels and enzyme activities of several important phase I cytochrome P450 enzymes (CYPs) and phase II enzymes were quantified under control conditions and compared with their levels of cryopreserved primary human hepatocytes as well as with their levels after specific

nuclear receptor activations (Chapter 3 and 4). These cryopreserved primary hepatocytes were reported to be representative for fresh primary hepatocytes with respect to their CYP and phase I enzymes activities towards a range of substrates [2].

The transcript levels of CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4 were measured with quantitative PCR. Results showed that transcripts of all CYPs were present in HepG2; however mRNA levels of most CYPs were significantly lower than in cryopreserved primary human hepatocytes. These results were confirmed with luminometric assays which were used to measure the enzyme activities of CYP1A1, 1A2, 2C9, and 3A4.

Regulation of CYP1A1, 1A2, 2B6, 2C8, 2D6, 2E1, and 3A4 by the aryl hydrocarbon receptor (AhR), pregnane X receptor (PXR) and constitutive androstane receptor (CAR) was studied in HepG2 cells at the mRNA and/or enzyme level. Regulation of CYP1A1, 1A2, 2B6, and 3A4 mRNA levels was similar to the regulation in primary human hepatocytes. In contrast, CYP2C8 mRNA levels are inducible in cryopreserved human hepatocytes, but not in HepG2 cells, after treatment with PXR/CAR activators. Consistent with other studies, CYP2D6 and 2E1 transcript levels were not changed after treatment with AhR, PXR, and CAR activators. Moreover, CYP1A1 and 1A2 enzyme activities could be induced by AhR agonists and CYP3A4 by PXR agonists.

Thus characterization of phase I CYPs showed that the enzymes were in general present in HepG2 cells, but at much lower levels than in cryopreserved primary human hepatocytes. Levels of these enzymes can be induced by activation of the AhR, PXR, and CAR.

Transcript levels of phase II UDP-glucuronosyl transferases (UGTs), sulfotransferases (SULTs), glutathione S-transferases (GSTs), N-acetyltransferase-1 (NAT1) and epoxide hydrolase (EPHX1) were measured with quantitative PCR in HepG2 cells and cryopreserved primary human hepatocytes. Levels of SULT1A1, 1A2, 1E1, 1A2, and 2A1, microsomal GST 1, GST μ1, NAT1, and EPHX1 in HepG2 cells were almost similar to levels in cryopreserved primary human hepatocytes. In contrast, levels of UGT1A1 and 1A6 transcripts were between 10- and more than 1000-fold higher in the cryopreserved primary hepatocytes. The regulatory processes of phase II enzymes by the AhR, PXR and CAR were studied in HepG2 cells and appeared quite similar to those in primary human hepatocytes. Due to the involvement of phase II enzymes in the toxification of several compounds, HepG2 cells can be a valuable cellular system to predict toxicity for these compounds.

The normal expression of most phase II enzymes in combination with the lower expression of CYPs in HepG2 cells might however result in an underestimation of toxicity for several compounds. Proximate genotoxins that need activation by low expressed CYPs (i.e. cyclophosphamide that is activated by CYP2B6) might be difficult to detect in HepG2 cells. The usage of S9 mixture or pre-stimulation of HepG2 cells with AhR,

PXR, or CAR agonists to induce CYP levels may be methods to solve these issues.

The development of a HCS in vitro micronucleus assay in the HepG2 and CHO-k1 cell line

To be able to rapidly assess chromosome damage (clastogenicity and aneugenicity) an automated image analysis assisted high content screening (HCS) in vitro micronucleus (IVMN) assay was developed using both the rodent CHO-k1 cell line commonly used in regulatory genotoxicity assays as well as the human HepG2 hepatoma cell line.

The reproducibility of the CHO-k1 and HepG2 HCS IVMN assays was tested with the genotoxic reference compound taxol and appeared to be good. The HCS IVMN assays were both evaluated by testing the ECVAM compound list. The sensitivity (number of genotoxic carcinogens correctly predicted) (80%; 16/20) and specificity (percentage of non-genotoxins correctly predicted) (88%; 37/42) of the CHO-k1 cell line were high. Proximate genotoxins that are activated by CYP1A (i.e. benzo[a]pyrene and 7,12-dimethylbenzanthracene) were already genotoxic in CHO-k1 cells without S9 mixture indicating that these enzymes are probably active in CHO-k1 cells. Addition of S9 mixture resulted even in a decrease in the genotoxic effects for these compounds.

The sensitivity of the HepG2 cell line was lower, being 60% (12/20), the specificity was high amounting to 88% (37/42). The metabolic capacity in hepG2 cells was able to detect genotoxic potential of compounds that need metabolic activity to exert their genotoxic activity. Seven out of the nine proximate genotoxins showed genotoxic potential, i.e. cyclophosphamide, benzo[a]pyrene, 7,12-dimethylbenzanthracene, dimethylnitrosamine, 2,4-diaminotoluene, PhIP.HCl and aflatoxin B1. The proximate genotoxins 2-acetylaminofluorene and IQ showed a negative result. The addition of S9 mixture did not result in activation of these two compounds.

Testing of an additional set of 16 genotoxic compounds confirmed results that were obtained with the ECVAM compound list. The sensitivity of the CHO-k1 cell line was 69% (11/16) and thus higher than the sensitivity of 56% (9/16) that was obtained for the HepG2 cell line. For both the CHO-k1 as well as HepG2 cell line it was possible to size-classify micronuclei enabling discrimination of aneugens from clastogens.

It was concluded that two high-throughput micronucleus assays were developed that can detect genotoxic potential (chromosome damage) and allow differentiation into clastogens and aneugens.

The identification of biomarkers for genotoxicity in HepG2 cells by gene expression profiling

Gene expression profiling in HepG2 cells was used to find biomarkers (genes and responsive elements) that can be used for the development of high-throughput luciferase based reporter assays that can assess the genotoxic potential of compounds and

discriminate genotoxic carcinogens from non-genotoxic liver toxicants.

HepG2 cells have been successfully used to discriminate genotoxic from non-genotoxic carcinogens by gene expression profiling. Classifiers that discriminated genotoxic and non-genotoxic carcinogens were involved in cell cycle regulation, cell cycle arrest, DNA damage related processes, immune and stress responses, and apoptosis. The focus in these studies was on the determination of genotoxic and non-genotoxic pathways involved in carcinogenesis and therefore in these studies non-toxic concentrations of the non-genotoxic carcinogens were used.

In the present study gene expression profiling in HepG2 cells was used to find genes and responsive elements that can not only assess the genotoxic potential of compounds but also discriminate genotoxic carcinogens from non-genotoxic liver toxicants. HepG2 cells were exposed to cytotoxic concentrations of the non-genotoxic liver toxicants as cytotoxicity can activate stress pathways, may cause DNA damage, and may thus result in a false-positive in vitro prediction for in vivo genotoxicity. The differences between the expression profiles of the genotoxic and liver non-genotoxic toxicants will be used to determine biomarker genes or responsive elements that are specific for genotoxins. Application of such biomarkers may result in a high-throughput luciferase based assay with a good sensitivity for genotoxicity and low false positive rate for non-carcinogens and in vivo genotoxicity.

HepG2 cells were treated with four genotoxic and seven non-genotoxic liver toxicants for 6h, 24h and 72h. Discrimination between the two classes of compounds was limited when all differentially expresses genes (DEG) were used. Pathway analysis of the DEG in the class of genotoxins revealed multiple affected pathways, of which the main ones at the three time points were involved in cell cycle control. At 72h several of the genotoxicity pathways were activated by the non-genotoxic liver toxicants (i.e. p53 pathway, ATM pathway, G2/M cell cycle checkpoint regulation).

Additional data analysis with Qlucore Omics Explorer was performed to find genes that differentiate both classes of compounds. This analysis yielded for each time point a subset of genes (6h, 362 genes; 24h, 1914 genes; 72h, 498 genes). Pathway analysis showed that the 6h and 24h gene subsets were mainly involved in the cell cycle, apoptosis, and DNA repair. No pathways were affected by the 72h subset of genes.

The most pronounced induced genes (top 10) in the three sets of genes were mainly downstream targets of p53, involved in apoptosis or the oxidative stress response. Several other pivotal genes involved in the DNA damage response were also present in the three subsets (i.e. GADD45A, GADD45B, PCNA, POLH, and XPC).

In summary, gene expression analysis with Qlucore Omics Explorer revealed a 6h, 24h, and 72h gene subset that discriminated genotoxic from non-genotoxic toxicants exerting their effect through cytotoxicity. The most prominent induced genes in the three subsets of genes were mainly involved in cell cycle control or were downstream targets

of p53. The application of these potential biomarkers in luciferase based reporter assays may provide a tool for screening genotoxicity with lower levels of false positives.

The development of luciferase based reporter assays for genotoxicity and oxidative stress

Gene expression analysis (Chapter 6) showed that the application of the promoters (or responsive elements) of genes involved in cell cycle control or of genes that are downstream targets of p53, might result in sensitive luciferase based reporter assays that can rapidly assess mammalian genotoxicity with a low rate of false positive results due to cytotoxicity. In addition, gene expression analysis indicated that RAD51C may be a specific marker for DNA double strand breaks.

Based on these data the promoter regions of RAD51C and cystatin A (downstream target of p53 involved in apoptosis), as well as the responsive element of the p53 protein, were selected for the generation of three genotoxicity reporter assays. Moreover, a luciferase-based reporter assay was generated that measures the activation of the Nrf2 electrophile responsive pathway (Chapter 7).

The reproducibility of the four reporter assays was tested with the genotoxic reference compounds doxorubicin and benzo[a]pyrene and appeared to be good for all four assays. Validation with respect to the ECVAM compound list resulted in an predictivity (total percentage correctly predicted genotoxic carcinogens plus non-genotoxic compounds) for the individual genotoxicity reporter assays of 82% for the HepG2 RAD51C_luc assay (sensitivity 60%, 12/20; specificity 93%, 39/42), 84% for the HepG2 Cystatin A_luc assay (sensitivity 70%, 14/20; specificity 90%, 38/42), and 90% for the HepG2 p53_luc assay (sensitivity 85%, 17/20; specificity 93%, 39/42). The overall predictivity of the three HepG2 reporter assays for genotoxicity was high with 82% (sensitivity 85%, 17/20; specificity 81%, 34/42). In addition, the percentage of genotoxic compounds that activated the Nrf2 pathway was high with 75% (15/20). Of the non-genotoxic compounds in the ECVAM list only 31% (13/42) activated the Nrf2 pathway.

The metabolic capacity in HepG2 cells was sufficient to activate seven out of the nine proximate genotoxins i.e. benzo[a]pyrene, 7,12-dimethylbenzanthracene, 2-acetylaminofluorene, 2,4-diaminotoluene, IQ, PhIP.HCl and aflatoxin B1. Of these seven compounds 2-acetylamininofluorene and IQ were not detected in the HepG2 HCS IVMN assay (Chapter 5) which might indicate that the activation of the p53 responsive element is a more sensitive endpoint for these compounds in HepG2 cells than the formation of micronuclei (DNA damage). The activation of the DNA repair response in HepG2 cells may have prevented a significant induction of micronuclei in the HepG2 HCS IVMN assay. The proximate genotoxins cyclophosphamide and dimethylnitrosamine were not detected in the genotoxicity reporter assays. As cyclophosphamide and dimethylnitrosamine are respectively activated by CYP2B6 and

2E1, which are expressed at low levels in HepG2 cells (Chapter 3), this low expression is a plausible reason for the fact that these two proximate genotoxins were not detected. However, this hypothesis is not supported by the fact that with the HepG2 HCS IVMN assay these compounds could be identified as genotoxic without the addition of S9 mixtures, implying that the endogenous enzyme levels are high enough for the detection in this assay. Therefore the formation of micronuclei might be a more sensitive endpoint for the detection of cyclophosphamide and dimethylnitrosamine. Notheworthy, in a study performed by Sohn et al. [3] there was also no p53 activation observed after exposure of cells to cyclophosphamide.

The data from the genotoxicity reporter assays were also compared with the available data on bacterial mutagenicity (Ames test), in vitro mammalian genotoxicity and in vivo genotoxicity for an additional set of 192 compounds. The predictivity for bacterial mutagenicity results was 74% (sensitivity 61%, 30/49; specificity 80%, 77/96) and for in vitro mammalian genotoxicity 59% (sensitivity 45%, 35/78; specificity 83%, 38/46). The correlation between results from the HepG2 genotoxicity reporter assays and in vivo genotoxicity was higher with 77% (sensitivity 74%, 28/38; specificity 81% 26/32). For the individual genotoxicity reporter assays, the correlations with in vivo genotoxicity were 76% for the HepG2 p53_luc assay (sensitivity 71%, 27/38; specificity 81%, 26/32), 59% for the HepG2 RAD51C_luc assay (sensitivity 29%, 11/38; specificity 94%, 30/32), and 76% for the HepG2 Cystatin A_luc assay (sensitivity 61%, 23/38; specificity 94%, 30/32). Like observed with the ECVAM compound list the HepG2 p53_luc assay and Cystatin A_luc assay are assays with a relative high sensitivity and specificity. The HepG2 RAD51C_luc assay has a lower sensitivity but the specificity is high indicating that only a few compounds induce double DNA strand breaks. Of the 108 compounds in the additional list of 192 compounds that have a positive result for bacterial or mammalian genotoxicity 62% (67 compounds) activate the Nrf2 pathway. This percentage was only 38% (30/80) in the group of compounds with negative of no genotoxicity data. These data confirm the results that were found with the ECVAM compound list that a large percentage of the genotoxic compounds activate the Nrf2 pathway. Activation of the Nrf2 pathway gives information about the mode of action of a genotoxic compound. This assay should however not be used to identify and subsequently deselect genotoxic compounds as this pathway is also activated by cytotoxic compounds and by various beneficial compounds that protect cells against genotoxic and cytotoxic compounds [4-6]. Activation of this pathway results in up-regulation of phase-II detoxifying enzymes and antioxidant-stress proteins. In addition, cross-talk between the p53 and Nrf2 pathways makes the individual role of Nrf2 in the genotoxic potential assessment as such very difficult [7].

In summary, three luciferase based reporter assays for the rapid assessment of genotoxic potential were generated. The p53 and cystatin A reporter assays had a high sensitivity

and specificity for genotoxic carcinogenicity and in vivo genotoxicity. The RAD51C reporter assay was a more specific assay that gave information about the formation of double strand breaks. The results of these individual assays supported the results that were found with gene expression profiling studies in HepG2 cells. In addition a Nrf2 reporter assay was developed that can help to elucidate the mode of action of a genotoxicant. Based on the obtained results with the different high-throughput reporter assays it can be concluded that application of these assays may be a useful strategy to rapidly assess genotoxic potential in the early discovery phase.

In this and the previous sections the focus was on the development and validation of assays for the detection of compounds with genotoxic potential. Besides the detection of genotoxic compounds (genotoxic carcinogens) it would also be useful to detect non-genotoxic carcinogens in the discovery phase of drug development. Non-genotoxic carcinogens can induce tumor formation by many different mechanisms [8]. One of the mechanisms is receptor mediated induction by the aryl hydrocarbon receptor which is focused on in the next section.

Detection of CYP1A inducers and species differences between human and rat

Strong activation of the AhR can lead to various toxic effects such as (non-genotoxic) carcinogenicity. Normally the majority of carcinogenicity studies are performed in rats, while for the prediction of human carcinogenicity human AhR activation should be studied.

CYP1A was used as marker for AhR activation as AhR activators induce this gene to high levels. CYP1A induction studies were performed in the human HepG2 and rat H4IIE cell lines. CYP1A induction in the HepG2 and H4IIE cell line has been shown to be representative for CYP1A induction in human and rat, respectively [2, 9, 10].

A set of 119 compounds, including known AhR ligands were tested. CYP1A induction was observed for 24 compounds. In H4IIE cells, more compounds resulted in induction and most EC50 values were below those of HepG2 cells. Species specific CYP1A induction in H4IIE and HepG2 cells was obtained for eight and three compounds, respectively.

Thus for several compounds species differences between CYP1A induction in human and rat were found. Therefore, given that possible subsequent carcinogenicity studies will be performed in rats but that risks should be determined for the human population, parallel screening in both species seems the best strategy in the pharmaceutical industry. Care should be taken to directly deselect compounds that are positive in these tests as

AhR activation does not necessarily indicate dioxin-like toxicity. There are for example marketed therapeutics like omeprazole, leflunomide, flutamide, and nimodipine which are safely used but have been proven to be AhR agonists [11]. Moreover, CYP1A induction by other pathways than the AhR is known, resulting in possible false positives in this assay [11]. In summary, both the human and rat CYP1A induction assays are useful for prescreening but care should be taken to deselect CYP1A inducers.

Comparison of the sensitivity and specificity of the regulatory and high-throughput in vitro genotoxicity assay

In the present thesis several high-throughput assays for the detection of genotoxic potential were evaluated. In this section the sensitivity and specificity of the newly developed high-throughput in vitro genotoxicity assays will be compared with the regulatory in vitro genotoxicity assays. Since in the current regulatory practice combinations of assays are used to detect bacterial mutagenicity (gene mutations) and mammalian genotoxicity (chromosome damage) a combined use of HTS assays will be discussed in this chapter. The sensitivity and specificity of the regulatory in vitro genotoxicity assay will be discussed first.

Sensitivity and specificity of combinations of regulatory in vitro genotoxicity assays

Kirkland et al. [12] evaluated the ability of single and combined use of the regulatory in vitro genotoxicity tests to discriminate rodent carcinogens and non-carcinogens. Hereby the authors stated that it is important to discriminate carcinogens into carcinogens that act via a genotoxic and non-genotoxic mode of action, as carcinogens that act via a non-genotoxic mode cannot be readily detected in in vitro genotoxicity assays [12]. Thus genotoxic carcinogens should give positive results in in vitro genotoxicity assay and non-genotoxic carcinogens and non-carcinogens should give negative results.

The performance scores of the individual in vitro genotoxicity assays are shown in Chapter 1 Table 3. These data clearly show that the specificity of the individual mammalian in vitro genotoxicity assays for non-carcinogens is relatively low resulting in a high percentage of false positives. In general a combination of the Ames test + MLA + chromosome aberration assay is used in regulatory testing [12] (Table 2). This combination has a sensitivity for carcinogenicity of 84.7% (171/202) but extremely low specificity for non-carcinogens of only 22.9 % (22/96). The combined use of two tests (i.e. Ames + CA, AMES + IVMN, AMES+MLA) gives similar scores [12]. The overlap between the compounds used by Kirkland et al. and the compounds used in the present thesis is however rather limited and therefore a straight comparison is not possible as

such. Also since mechanistic data was lacking for most carcinogens it was not possible for Kirkland et al. to calculate the performance scores for genotoxic carcinogenicity [12].

The assess the sensitivity and specificity of the proposed in vitro models, as shown in the present thesis an 'ECVAM compound list' was used [13]. This list consists of well defined genotoxic carcinogens, non-genotoxic carcinogens and non-carcinogens. The compound list was used to calculate the sensitivity and specificity for genotoxic carcinogenicity (Table 2). Application of the regulatory accepted test strategy (combination of Ames + MLA + CA test) gives in case of these ECVAM compounds a sensitivity for genotoxic carcinogenicity of 100% (20/20) and specificity of 55% (23/42). The low specificity is mainly caused by the in vitro mammalian genotoxicity assays (MLA + CA) that give 19 false positive results.

Besides the ECVAM compounds an additional list of 192 compounds was used in the present thesis (Chapters 2 and 7). Genotoxicity and carcinogenicity data are limited for these compounds and a clear classification in genotoxic carcinogens and non-genotoxins (non-genotoxic carcinogens + non-carcinogens) is not available. For these compounds we made therefore the choice to compare the assay data with in vivo genotoxicity data, of which the result are most likely the best reflection of the discrimination between genotoxic carcinogens and non-genotoxins. With the use of this additional compound set, the combination AMES + in vitro mammalian assays (Table 2) shows a sensitivity of 97% (37/38) and specificity of 41% (12/29). Also here the low specificity is caused by the results from the in vitro mammalian genotoxicity assays (Table 2).

Overall the two compound sets that were used in the present thesis give, although the way of comparing was different, similar results as shown in the Kirkland study [12]. A combination of the AMES + in vitro mammalian genotoxicity assays gives a high sensitivity but low specificity. The ECVAM and additional compound list make a direct comparison between results from regulatory in vitro genotoxicity tests (Ames + in vitro mammalian assays) and the high-throughput assays developed in the present thesis possible. Different combinations of the high-throughput assays will be discussed below and the scores will be compared with those of the regulatory in vitro genotoxicity assays.

Sensitivity and specificity of combinations of the HTS genotoxicity assays and comparison with the regulatory in vitro screening battery

Taking as starting point that a screening battery for early genotoxicity screening should contain a bacterial based assay to be able to detect gene mutations, the Vitotox™ assay should be present. The high sensitivity and specificity of the Vitotox™ for Ames results of respectively more than 80% and 90% (Chapter 2), the high-throughput and low amount of compound needed makes this assay a valuable prescreen that can rapidly

predict Ames test results in the discovery phase of drug development.

Besides bacterial mutagens, an early screening battery should contain assays that are able to detect compounds causing chromosome damage. Several assays that have the potential to detect chromosome damage were validated in the present study, Combinations of these assays, i.e. (1) the yeast based RadarScreen assay, (2) the CHO-k1 HCS IVMN assay, (3) the HepG2 HCS IVMN assay, and (4) the luciferase based p53 reporter assays in HepG2, with the Vitotox™ are discussed below. The sensitivity and specificity scores of the combinations of assays are summarized in Table 3.

1. Vitotox + RadarScreen

In case of the ECVAM compounds the sensitivity (80%) and specificity (81%) is high. However, in case of the additional list of compounds the specificity score is low with only 28%. Therefore this combination of tests is difficult to use in the early discovery phase as too many pharmacologically interesting compounds will be deselected based on a false positive result.

2. Vitotox + CHO-k1 HCS IVMN assay

This combination gives in case of the ECVAM compounds a sensitivity of 95% (19/20). The specificity of the combination Vitotox™ + CHO-k1 HCS IVMN is with 83% (35/42) much higher than the specificity of 55% (23/42) of the combination regulatory Ames + in vitro mammalian genotoxicity assays. These performance scores make the combination Vitotox™ + CHO-k1 HCS IVMN assay applicable for early screening.

Additional validation may be essential as the regulatory IVMN assay in which also often CHO-k1 cells are used gives a much lower specificity (30.8% for non-carcinogens [12]). Reasons for the differences between the present and previous studies might be the presence of a functionally active p53 protein (non published studies with the p53 inhibitor pifithrin-α show that p53 is at least partly functional in the CHO-k1 cell line used) and/or the application of the HCS technique. This HCS technique gives promising results and seems to be more consistent and reliable also due to the lack of observer variability that is seen with manual scoring of micronuclei [14].

3. Vitotox + HepG2 HCS IVMN assay

A combination of these assays gives in case of the ECVAM compounds a sensitivity of 90% (18/20) and specificity of 83% (35/42). Similar to the HCS IVMN assay in CHO-k1 cells, the specificity of the HepG2 HCS IVMN assay is considerably higher (83%) than the specificity of the regulatory Ames + in vitro mammalian genotoxicity assays. The observed sensitivity and specificity scores indicate that application of a combination of these two assays in the discovery phase will be a useful test strategy.

Table 2. Sensitivity and specificity scores (of combinations) of regulatory in vitro genotoxicity assays for three compound sets.

Compound set	**Kirkland et al. [12]**	**Kirkland et al. [13] (ECVAM)**	**Current thesis (additional compound list)**		
Endpoint	**Carcinogenicity**	**Genotoxic carcinogenicity**	**In vivo genotoxicity**		
Tests	1. Ames 2. in vitro mammalian gen. assays	1. Ames 2. in vitro mammalian gen. assays	1. Ames	1. in vitro mammalian gen. assays	1. Ames 2. in vitro mammalian gen. assays
Sensitivity %	84.7 (171/202)	100 (20/20)	48 (16/33)	94 (34/36)	97 (37/38)
Specificity %	22.9 (22/96)	55 (23/42)	79 (22/28)	46 (12/26)	41(12/29)

Table 3. Performance scores of combined use of high-throughput genotoxicity assays presented in the current thesis. The scores are shown for the additional list and/or ECVAM compound list.

Test combination	**1. Vitotox™ 2. RadarScreen**		**1. Vitotox™ 2. CHO-k1 IVMN**	**1. Vitotox™ 2. HepG2 IVMN**	**1. Vitotox™ 2. HepG2 p53_luc**	
Compound set name	ECVAM	Additional	ECVAM	ECVAM	ECVAM	Additional
Sensitivity (%)	80 (17/20)	73 (27/37)	95 (19/20)	90 (18/20)	85 (17/20)	76 (29/38)
Specificity (%)	81 (34/42)	28 (9/32)	83 (35/42)	83 (35/42)	88 (37/42)	72 (23/32)

4. Vitotox + HepG2 p53_luc

This combination of assays gives in case of the ECVAM compounds a sensitivity of 85% (17/20) and specificity of 88% (37/42). Only three genotoxic carcinogens are not detected and the specificity of 88% is much higher than the specificity of 55% (23/42) with the regulatory Ames + mammalian in vitro genotoxicity assays.

For the additional set of compounds a sensitivity and specificity of respectively 76% (29/38) and 72% (23/32) is measured. These sensitivity scores appeared to be lower than those of the Ames + in vitro mammalian genotoxicity assays combination (97%, 37/38) but the specificity is with 72% much higher than the 41% (12/29) measured with the Ames + in vitro mammalian genotoxicity assays combination. Overall the results show that a combination of Vitotox™ + HepG2 p53_luc gives a high sensitivity and high specificity which make this combination most suitable for early screening. An additional advantage of the tested combination of these two assays is that the validation studies were performed with a large set of compounds (ECVAM + additional set).

Combinations of the Vitotox™ with the cystatin A and RAD51C reporter will not be discussed as both these genes are p53 dependent for their transcription [15]. Therefore these assays are not essential to perform and do have (almost) no effect on the performance scores (Chapter 7). Thus of these three reporter assays, the p53 assay is the most useful for the detection of genotoxic potential. Additional reporter assays like cystatin A, RAD51C, but also Nrf2 give information about the mode of action and can therefore also be of use. Addition of these assays in the test strategy does however not result in a decrease of specificity or an increase of sensitivity.

Thus in summary, four combinations of HTS assays were evaluated. The combination Vitotox™ + RadarScreen resulted in a specificity that is generally too low. The other three combinations of assays (Vitotox™ + CHO-k1 HCS IVMN assay, Vitotox™ + HepG2 HCS IVMN assay, and Vitotox™ + HepG2 p53_luc) seem all to be useful for screening in the early discovery phase as both the sensitivity and specificity are high. The specificity of all three combinations is higher than the combination regulatory Ames + mammalian in vitro genotoxicity assay. Of the three combinations the Vitotox™ + HepG2 p53_luc test strategy is the one who could be validated with the largest compound set, which resulted overall in the highest confidence during its use. Therefore at present this combination is preferably be used for an early screening strategy.

Further validation of the HCS IVMN assays with a larger compound set is proposed. Depending on the results a HCS IVMN assay may be added to the Vitotox™ + p53_luc combination which will make a direct discrimination of genotoxins between aneugens and clastogens possible in the near future.

Conclusion

In the present thesis several high-throughput assays to detect genotoxic and non-genotoxic carcinogenic potential were developed and validated with proper reference compounds. The results in this thesis show that an early prediction can be made for bacterial mutagenicity (gene mutations), mammalian genotoxicity (chromosome damage), and non-genotoxic carcinogenic potential by AhR activation.

To develop a strategy for application of the HTS genotoxicity assays in the early discovery phase several combinations of assays were evaluated. The combination Vitotox™ + HepG2 p53 reporter assay was based on the presented results in this thesis the most useful for screening compounds for their genotoxic potential in the early drug discovery phase without the risk on high numbers of false positives. CYP1A induction assays in human HepG2 and rat H4IIE cells may be performed in parallel with these assays to be able to detect non-genotoxic carcinogenic potential by AhR activators. Further application of these assays may prove useful in future drug development strategies.

Additional considerations

The regulatory genotoxicity assays and genotoxicity assays for early screening are in general performed in serial order. For each individual assay compound stocks are ordered and from these stocks dilution series are prepared. This strategy is relatively laborious, needs a high amount of compound and is especially a problem for screening in the discovery phase where the number of compounds is high and the amount of compound available is limited. The strategy of parallel screening may be more useful for early screening [16]. In parallel screening, one compound stock plate with dilution series is prepared and used to add the compound dilutions (by using a robotic device) to assay plates from different assays that are performed in parallel. This strategy saves compound and results in a quick generation of assay data.

Another important determinant for the success of early screening is the purity of compounds. In the early research phase there is a balance between the quick preparation of new chemical entities and the extent of impurities. In pharmacological testing small amounts of impurities are often not a problem due to the concentrations used however as the concentrations used in the genotoxicity assays go up to a high concentration of 10^{-3} M, genotoxic impurities that are present in only low amounts might generate false positive results. Therefore the purity of the compounds should be high enough to generate not too much false positive results. On a case by case base it is for pharmacologically interesting compounds that show genotoxic potential possible to purify compounds by

crystallization or HPLC separation.

The application of an S9 metabolic system in the HepG2 and CHO-k1 cell lines needs also some consideration. Although we (Chapter 5) and others [17] showed that it is possible to use an S9 metabolic system in combination with HepG2 and CHO-k1 cells to activate proximate genotoxins like aflatoxin B1, the results in the present thesis showed also that the application of S9 mixture in bacteria and yeast gives sometimes different results than in CHO-k1 and HepG2 cells. In bacteria and yeast proximate genotoxins were not active without S9 mixture and addition of S9 mixture resulted in activation. This was also the case for HepG2 and CHO-k1 cells when there was no or very limited activation by endogenous metabolism. When however there was already a strong activation without S9 mixture, addition of S9 mixture resulted in a decrease of the genotoxic effects. The bacteria and yeast that were used in the present thesis contain genetic modifications that make the membranes more permeable so that genotoxic metabolites that are formed outside the cell by S9 mixture can easily enter the cells and reach the DNA. Moreover, in bacteria the compounds have to pass only one membrane to reach the DNA. In the human HepG2 (and rodent CHO-k1) cell line the hydrophilic metabolites might enter the cells slower than the parent compound. This might result in a lower amount of reactive metabolites reaching the DNA than in the case where the parent compound is activated endogenously within the cells.

Another consideration to be discussed is the method of validation. The validations performed in the present study gave a first indication that the methods developed are reproducible. These validations were however not yet performed according to the validation process defined by the ECVAM which would ultimately be required if one would aim at regulatory acceptance of the tests. This way of validation is a long lasting process that consists of a lot of steps (http://tsar.jcr.ec.europa.eu/), including method submission, regulatory relevance assessment, pre-validation, validation, peer review of results, validation statement, post-validation and finally the regulatory approval process. The strength of the current assays is the applicability in the early phase of drug development, and for this application an official validation is not needed. However, finally for some of the presented assays like for example the p53 reporter assay in HepG2 cells it might be useful to get regulatory acceptance.

The last consideration is about additional assays that might be useful to develop in the future. Gene expression analysis with genotoxic and non-genotoxic toxicants revealed several biomarker genes that discriminated genotoxic from non-genotoxic toxicants exerting their effect through cytotoxicity. Based on these data three luciferase based genotoxicity reporter assays were developed. Two of these luciferase based reporter assays appeared to be useful for the identification of compounds with genotoxic potential (p53 and cystatin A reporter assays). The third assay being the RAD51C reporter assay was more specific and identified the mode of action of genotoxins, being the generation

of DNA double strand breaks. Additional gene expression profiling studies in HepG2 cells may result in the identification of more biomarker genes that can discriminate the mode of action of a genotoxin. It would be useful to finally have a set of biomarkers that can discriminate direct acting genotoxins, cross-linking agents, topoisomerase inhibitors, DNA synthesis inhibitors, reactive oxygen species generators and aneugens. Such biomarker genes may be used for the development of high-throughput assays like luciferase based reporter assays. As proposed in this thesis high-throughput assays that can detect the non-genotoxic carcinogenic potential of compounds in the early discovery phase of drug development might be useful. We presented the development of an assay that is able to detect compounds that cause non-genotoxic carcinogenicity by activation of the AhR. This is however only one of many mechanisms that cause non-genotoxic carcinogenicity. Activation of several other receptors than the AhR can also result in non-genotoxic carcinogenicity. For such receptors like for example the peroxisome proliferator activated receptor α, CAR, thyroid hormone receptor it might therefore also be useful to develop screenings assays in human and/or rodent cell lines.

All together the results of the present thesis present several newly developed genotoxicity assays and demonstrate that these new assays may provide a reliable and useful high-throughput alternative for regulatory genotoxicity tests. Especially a combination of some of the newly developed test was shown to provide excellent possibilities for screening compounds for their genotoxic potential in the early drug discovery phase without the risk on high numbers of false positives. In addition, two assays to detect non-genotoxic carcinogenic potential by AhR activators in human and rat were presented. Several of the newly developed assays in this thesis may prove useful in future drug development strategies.

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Chapter 10

Samenvatting, discussie, conclusie en additionele opmerkingen

Achtergrondinformatie

De investeringen van de farmaceutische industrie in het onderzoek naar en de ontwikkeling van geneesmiddelen zijn de afgelopen decennia fors gestegen. Ondanks deze toename in R&D kosten, het gebruik van nieuwe test- en meettechnieken, automatisering en robotisering is het aantal nieuwe geneesmiddelen dat per jaar op de markt komt afgenomen.

Toxiciteit is in 30 tot 40% van de gevallen de oorzaak voor het feit dat potentiële geneesmiddelen niet verder ontwikkeld kunnen worden. Lever-, nier-, hart- en reproductie-toxiciteit, embryotoxiciteit, genotoxiciteit en carcinogeniciteit zijn de belangrijkste oorzaken.

Het vroegtijdig detecteren van toxiciteit in de eerste onderzoeksfase (discovery) van het geneesmiddelenonderzoek kan er uiteindelijk toe leiden dat geneesmiddelen met verbeterde profielen de latere fase van het geneesmiddelenonderzoek bereiken. Het onderzoek dat beschreven wordt in dit proefschrift, richt zich op de detectie van genotoxiciteit en carcinogeniciteit in deze vroege fase van het geneesmiddelen onderzoek.

Om te onderzoeken of potentiële geneesmiddelen veilig zijn, moeten deze voor de regelgevende (regulatoire) autoriteiten getest worden op o.a. genotoxiciteit en carcinogeniciteit. Het vaststellen van genotoxische eigenschappen van kandidaat-geneesmiddelen en het eventueel onderzoeken of deze effecten schadelijk kunnen zijn voor de mens, gebeurt in een gefaseerde aanpak. Als eerste worden cellulaire *in vitro* assays uitgevoerd om te onderzoeken of de testverbindingen intrinsieke genotoxische activiteit hebben. Deze *in vitro* testen worden opgevolgd door *in vivo* testen (dierproeven) die de relevantie van deze *in vitro* testen voor de mens vaststellen.

De teststrategie die voorgeschreven wordt door de regelgevende autoriteiten bestaat uit een combinatie van testen die samen zowel genmutaties als chromosoomschade (toename v.h. aantal chromosoombreuken, clastogeniciteit, en verandering van het aantal chromosomen per cel, aneugeniciteit genoemd) kunnen vaststellen. De standaard genotoxiciteitstesten die voor de regelgevende autoriteiten uitgevoerd moeten worden, voordat geneesmiddelen op de markt toegelaten worden, zijn (1) de Amestest voor het identificeren van genmutaties in bacteriën, (2) een *in vitro* chromosoomaberratietest (CA) en/of een 'muize-lymphoma-TK-assay (MLA)' om chromosoomschade in zoogdiercellen te detecteren. Deze testen worden dan eventueel opgevolgd door (3) een *in vivo* test die chromosoomschade kan detecteren (een CA- of micronucleustest). Eventueel kunnen aanvullende testen nodig zijn. Voor de bepaling van de mogelijke carcinogene werking van een teststof, worden muizen of ratten chronisch (twee jaar) blootgesteld aan deze teststof en wordt de vorming van tumoren gevolgd en geanalyseerd. De testen om

de eventuele genotoxiciteit van teststoffen vast te stellen, worden uitgevoerd in de preklinische fase van het ontwikkelingstraject van een nieuw geneesmiddel voordat de eerste klinische testen op mensen uitgevoerd worden. De mogelijke carcinogeniciteit wordt later onderzocht, parallel aan klinische fase II- en III-studies.

De regulatoire testen voor het vaststellen van mogelijke genotoxiciteit en carcinogeniciteit hebben een lage doorvoer snelheid (throughput), verbruiken relatief veel teststof en zijn arbeidsintensief. Vanwege deze eigenschappen zijn de regulatoire testen minder geschikt voor het snel testen van genotoxiciteit en carcinogeniciteit in de discovery fase van het geneesmiddelenonderzoek.

De afgelopen jaren is er binnen de farmaceutische industrie al enige vooruitgang geboekt met de ontwikkeling van testsystemen voor het bepalen van genotoxische en carcinogene eigenschappen aan het einde van de discovery fase. Echter aan het einde van de discovery fase is chemische optimalisatie van teststoffen op zowel farmacologische activiteit, als op ongewenste eigenschappen moeilijk te realiseren. Voor deze toepassing moeten de testen dus in de vroegere fase van de discovery toegepast worden.

Momenteel zijn er echter nog maar enkele testsystemen beschikbaar voor het bepalen van genotoxiciteit en carcinogeniciteit in de vroege discovery fase, de doorvoer snelheid van deze testen is over het algemeen nog te laag en meestal zijn deze testen nog niet afdoende gevalideerd. Een belangrijk aspect is dat de huidige *in vitro* testsystemen voor de detectie van genotoxiciteit een hoog percentage fout-positieve resultaten geven (zie tabel 1). Dit maakt de toepassing van deze testen in de vroege fase van geneesmiddelenontwikkeling problematisch.

Het doel van het onderzoek in dit proefschrift is betere *in vitro* testsystemen te ontwikkelen voor de vroege detectie van de genotoxische en niet-genotoxische carcinogene eigenschappen van potentiële geneesmiddelkandidaten en deze testen te valideren met referentieverbindingen en uiteindelijk een strategie op te zetten voor de toepassing van deze testen in de vroege discovery fase. In dit proefschrift zijn testsystemen op basis van bacteriën, gist-, knaagdier- en menselijke cellen ontwikkeld en gevalideerd. Bij de humane cellen lag de focus op het gebruik van (lever hepatoma) HepG2 cellen, omdat een aantal gunstige eigenschappen van deze cellen, zoals een actief p53 eiwit, actief DNA herstel, en het bezit van metaboliserende enzymen, er toe zal bijdragen dat deze cellen een nauwkeuriger voorspelling geven voor *in vivo* genotoxiciteit (hoge gevoeligheid (sensitiviteit), laag percentage fout positieve resultaten).

Dit hoofdstuk geeft een samenvatting en discussie van de resultaten van dit proefschrift. Het eerste deel geeft een overzicht, op basis van hoofdstuk 2, van de resultaten die verkregen zijn met de bacteriële reporter assay Vitotox™. Deze test werd gevalideerd als snelle prescreen voor de regulatoire Amestest. Naast testen die in staat zijn om genmutaties te detecteren (bacteriële mutageniciteit) zou een prescreen voor het vaststellen van genotoxiciteit ook teststoffen moeten kunnen detecteren

die chromosoombeschadigingen veroorzaken (clastogeniciteit en aneugeniciteit). Om die reden zijn ook modelsystemen in gist en zoogdiercellen geëvalueerd. In het tweede deel van deze samenvatting wordt op basis van de hoofdstukken 2 en 7 een overzicht gegeven van de resultaten die verkregen zijn met de gist-RAD54-reporter assay. In het derde deel wordt een overzicht gegeven van de resultaten die verkregen zijn met de HepG2 cellijn (hoofdstukken 3, 4, 5, 6 en 7). Dit derde deel start met een beschrijving van de resultaten van de karakterisering van het cytochroom P450 en fase-II- metabolisme in HepG2-cellen (hoofdstukken 3 en 4). Vervolgens worden de resultaten besproken betreffende de ontwikkeling en validatie van twee high content screening (HCS) *in vitro* micronucleus(IVMN)-assays in de HepG2-cellen en CHO-k1-cellen (hoofdstuk 5). De CHO-k1-cellen werden als referentiecellijn meegenomen in het validatieproces omdat deze cellen gebruikt worden in de regulatoire IVMN-test. Vervolgens worden de de resultaten beschreven van een genexpressieprofileringstudie in HepG2-cellen. Deze studie werd gebruikt om gevoelige en specifieke biomarkers te vinden voor genotoxiciteit (hoofdstuk 6). Op basis van deze studie zijn luciferase reporterassays in HepG2-cellen ontwikkeld en geëvalueerd (hoofdstuk 7). Het derde gedeelte van de samenvatting wordt afgesloten met een beschrijving van deze resultaten. Naast het uitvoeren van testen die stoffen met genotoxische eigenschappen kunnen detecteren, is het ook nuttig om stoffen met een niet-genotoxisch carcinogene werking te detecteren in de discovery fase van de geneesmiddelenontwikkeling. Deel vier van deze samenvatting beschrijft daarom op basis van hoofdstuk 8 één van de mechanismen die niet-genotoxische carcinogeniciteit kan veroorzaken, namelijk de activatie van de aryl-hydrocarbon-receptor (AhR). De verschillen tussen de activering van deze receptor in de rat en in de mens werd onderzocht. Na een samenvatting van de resultaten van alle individuele testen zullen tot slot combinaties van testen worden besproken in relatie tot de ontwikkeling van een strategie voor het gebruik van de testsystemen in de discovery fase van het geneesmiddelenonderzoek. De samenvatting wordt afgesloten met een algemene conclusie, gevolgd door een aantal additionele opmerkingen.

Het bepalen van genotoxiciteit met de bacteriële Vitotox™-test

Normaal gesproken wordt bacteriële mutageniciteit met de Amestest bepaald, waarbij sprake is van een lage throughput. Een alternatief voor de detectie van bacteriële mutageniciteit zou een test kunnen zijn waarbij de inductie van genen wordt gemeten die betrokken zijn bij de reparatie van bacterieel DNA (SOS response). Door deze activatie fluorometrisch of luminometrisch te meten kan een test met een hoge throughput ontstaan. Een test die is gebaseerd op dit principe is de Vitotox™-test.

Tabel 1. Overzicht van de reguliere in vitro genotoxiciteitstesten en de alternatieve high-throughput modelsystemen die in dit proefschrift worden geëvalueerd.

Eindpunt in de assay	Regulatoire test(s)	Sensitiviteit	Specificiteit	High-throughput modelsystemen
1. Bacteriële mutageniciteit (gen mutaties)	Ames	58.5% (318/541)	73.9% (130/176)	Vitotox™ reporter assay (hoofdstuk 2)
2. Genotoxiciteit in zoogdiercellen (chromosoom schade: clastogeniciteit en aneugeniciteit)	MLA + CA	81.3% (165/203)	27.1% (26/96)	RadarScreen-assay in gist (hoofdstuk 2) CHO-k1-HCS-IVMN (hoofdstuk 5) HepG2-HCS-IVMN (hoofdstuk 5) HepG2 luciferase reporter assays (hoofdstuk 7)
1 + 2	Ames + MLA + CA	84.7 % (171/202)	22.9% (22/96)	

Afkortingen: HCS=high content screening; IVMN=in vitro micronucleustest; MLA=muize lymphoma TK assay

Deze Vitotox™-test is een bacteriële reporter assay in *Salmonella typhimurium*. In de genetisch gemodificeerde bacteriestam die gebruikt wordt in de Vitotox™-assay resulteert activatie van de SOS-response in een verhoogde concentratie luciferase die luminometrisch gemeten kan worden. In hoofdstuk 2 van dit proefschrift wordt de Vitotox™ geëvalueerd als vroege bepaling voor bacteriële mutageniciteit.

Een door de ECVAM aanbevolen lijst met teststoffen werd gebruikt voor de validatie van de Vitotox™ en andere genotoxiciteitstesten in dit proefschrift. Deze lijst met teststoffen bestaat uit 20 genotoxische carcinogenen en 42 niet-genotoxische chemicaliën. Van de 20 genotoxische carcinogenen veroorzaken 14 teststoffen bacteriële mutageniciteit (positief resultaat in de Amestest). De resterende 6 veroorzaken chromosoombeschadigingen, maar geven negatieve of niet eenduidige resultaten in de Amestest. De groep van 42 niet-genotoxische teststoffen (niet-genotoxische niet-carcinogene teststoffen en niet-genotoxische carcinogenen) bevat 19 verbindingen die fout-positieve resultaten geven in *in vitro* genotoxiciteitstesten in zoogdiercellen. Bovendien werd een extra set van 192 teststoffen gebruikt voor de verdere validatie van de high-througput testen. Deze groep van 192 teststoffen bevat zowel genotoxische als niet-genotoxische verbindingen en bevat zowel teststoffen van de voormalige NV Organon alsook bekende referentieverbindingen. De lijst bevat bovendien een relatief groot aantal steroïdale teststoffen waarvan beschreven is dat ze clastogene en/of aneugene eigenschappen hebben. Van deze 192 teststoffen waren Amesgegevens beschikbaar voor 145 teststoffen, *in vitro* genotoxiciteitsdata in zoogdiercellen voor 124 teststoffen en *in vivo* genotoxiciteitsdata voor 70 teststoffen. De sensitiviteit (percentage van de genotoxische carcinogenen die een positief resultaat geven) van de Vitotox™-test met betrekking tot de ECVAM stoffenlijst was 70% (14/20). Zonder de teststoffen die een negatief of onduidelijk resultaat in de Amestest geven, steeg de sensitiviteit naar 79% (11/14). De specificiteit (percentage van niet-genotoxische verbindingen die negatief resultaat geven) van de Vitotox™-test was met 93% (39/42) hoog. De resultaten met betrekking tot de extra lijst van 192 stoffen bevestigden de resultaten die gemeten werden met de ECVAM-lijst. De validatie met deze extra stoffen liet zien dat de resultaten van de Vitotox™ een hoge correlatie met de resultaten van de Amestest hebben van 91% (sensitiviteit, 86% (42/49); specificiteit, 94% (90/96)). De correlatie met *in vitro* genotoxiciteitstesten in zoogdiercellen en *in vivo* genotoxiciteit was veel lager met respectievelijk 50% (sensitiviteit 27% (21/78); specificiteit 89% (41/46)) en 54% (sensitiviteit 33% (13/38); specificiteit 78% (25/32)).

Samengevat lieten de resultaten in hoofdstuk 2 zien dat de Vitotox™-test een goede methode is om de resultaten van de Amestest mee te voorspellen. De Vitotox™-test gaf vergelijkbare resultaten als de Amestest. De hoge doorvoersnelheid van de test (16 verbindingen per dag) en lage hoeveelheid teststof die nodig is (5 mg) resulteren erin dat de Vitotox™-test geschikt is voor het testen in de vroege fase van het geneesmiddelenonderzoek. Ter vergelijking: zelfs geminiaturaliseerde versies van de

Amestest hebben nog steeds 300 mg testverbinding nodig en de throughput van de mini-Ames is minder dan 5 teststoffen per week [1]. Dit heeft als gevolg dat de mini-Amestest wordt uitgevoerd op een later tijdstip in de drug discovery fase, waarbij optimalisatie op zowel farmacologische eigenschappen, als op genotoxiciteit moeilijk te verwezenlijken is. De resultaten met de Vitotox™ laten ook duidelijk zien dat voor de detectie van chromosoomschade andere modelsystemen vereist zijn.

Het bepalen van genotoxiciteit met de op gistcellen gebaseerde RadarScreen-assay

Om chromosoomschade te kunnen detecteren zijn modelsystemen in eukaryote cellen nodig. Een modelsysteem dat gebaseerd is op het gebruik van eukaryote cellen en dat werd gevalideerd in hoofdstuk 2 van dit proefschrift is de op gistcellen gebaseerde RadarScreen-test. De RadarScreen-test maakt gebruik van een β-galactosidase reportergen dat onder controle staat van een RAD54-promoter. De β-galactosidase expressie kan luminometrisch gekwantificeerd worden. RAD54 speelt een centrale rol in het herstel van DNA-schade in gist en activatie van dit gen leidt in deze assay tot een lichtreactie (luminescentie), die daarom een maat is voor de mate van DNA schade.

De reproduceerbaarheid van de test werd getest met de genotoxische referentiestoffen methyl-methaan-sulfonaat en benzo[a]pyrene en bleek hoog te zijn. Validatie met betrekking tot de ECVAM-stoffenlijst resulteerde in een correlatie met (*in vivo*) genotoxiciteit van 79% (sensitiviteit, 70% (14/20); specificiteit 83% (35/42)). Voor de extra set van 192 verbindingen, had de RadarScreen een correlatie met *in vitro* genotoxiciteit in zoogdiercellen van 76% (sensitiviteit, 77% (59/77); specificiteit, 74% (34/46)). De resultaten met de ECVAM-stoffenlijst gaven de indicatie dat het aantal foutpositieve resultaten met deze test waarschijnlijk laag is. Dit werd echter niet bevestigd met de aanvullende lijst van 192 teststoffen. Met deze teststoffen bleek de predictiviteit voor *in vivo* genotoxiciteit slechts 54% (sensitiviteit, 68% (25/37); specificiteit, 38% (12/32)). Vooral de steroïdale verbindingen in de aanvullende lijst gaven veel foutpositieve resultaten voor *in vivo* genotoxiciteit. De exacte reden voor deze resultaten is niet bekend. Een mogelijke oorzaak voor de lage voorspelbaarheid zou een verschil in metaboliserende enzymen kunnen zijn geweest. Bovendien bleek de detectie van aneugenen moeilijk met de RadarScreen-test.

Samenvattend kan worden gesteld dat met de RadarScreen-test een goede voorspelbaarheid voor *in vitro* genotoxiciteit in zoogdiercellen werd gemeten maar dat de voorspelbaarheid van de test voor *in vivo* genotoxiciteit laag is. Om die reden zijn betere modelsystemen noodzakelijk.

Het bepalen van genotoxiciteit met de humane HepG2-cellijn

Een nadeel van het gebruik van de meeste permanente cellijnen is dat ze geen functioneel p53 eiwit, DNA-reparatierespons en metaboliserende capaciteit bezitten. Een gebrek aan functionaliteit in deze systemen zou de reden kunnen zijn voor de lage voorspelbaarheid van de meeste *in vitro* modelsystemen voor *in vivo* genotoxiciteit. Het gebruik van de menselijke hepatoma cellijn HepG2 zou daarom nuttig kunnen zijn omdat deze cellen een actief p53-eiwit, DNA-reparatiesystemen en fase I- en II-metaboliserende enzymen bevatten.

In dit proefschrift zijn de volgende studies met deze cellijn uitgevoerd: (1) fase I- en II-metabolisme werd gekarakteriseerd (hoofdstukken 3 en 4), (2) een high content screening (HCS) *in vitro* micronucleus(IVMN)test werd ontwikkeld en gevalideerd in HepG2-cellen evenals in CHO-k1-cellen. Deze CHO-k1-cellijn wordt veelvuldig in de in de regulatoire IVMN-test gebruikt (hoofdstuk 5), (3) genexpressieprofielen werden geanalyseerd in HepG2-cellen die blootgesteld werden aan genotoxische- en niet-genotoxische levertoxische stoffen. De resultaten werden gebruikt om biomarkers voor genotoxiciteit te bepalen (hoofdstuk 6), (4) en ten slotte zijn op basis van deze genexpressieprofielen luciferase reporter assays voor de detectie van genotoxische teststoffen ontwikkeld en gevalideerd (hoofdstuk 7).

Karakterisering van fase I- en II-metabolisme in de HepG2-cellijn

Metabolisme van teststoffen door fase I- en II-enzymen kan resulteren in de vorming van metabolieten die juist meer of minder toxisch zijn dan de teststoffen zelf. Kennis van de metabole status van cellijnen die gebruikt worden voor *in vitro* toxiciteitstesten is daarom een belangrijke factor om de resultaten van *in vitro* (geno-) toxiciteitstesten te begrijpen en de waarde deze testen te onderkennen. Om deze redenen werd in dit proefschrift het fase I- en II-metabolisme in de HepG2- cellijn gedetailleerd gekarakteriseerd. De basale messenger-RNA(mRNA)-niveaus en enzymactiviteiten van een aantal belangrijke fase I-cytochroom P450 (CYP) en fase II-enzymen werden gemeten en vergeleken met de mRNA-niveaus en -activiteiten in gecryopreserveerde primaire humane hepatocyten (levercellen). Daarnaast zijn de mRNA-niveaus en enzymactiviteiten ook gemeten na activatie van specifieke nucleaire receptoren (hoofdstuk 3 en 4). De gecryopreserveerde primaire hepatocyten die werden gebruikt in de studies hadden vergelijkbare enzymactiviteiten als verse primaire hepatocyten [2].

De mRNA-niveaus van CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 en 3A4 werden gemeten met kwantitatieve PCR (qPCR). De resultaten toonden aan dat mRNA's van al deze CYP's aanwezig waren in HepG2. Maar de mRNA-niveaus van de meeste CYP's waren significant lager dan de niveaus in gecryopreserveerde primaire humane

hepatocyten. Deze resultaten werden bevestigd met luminometrische bepalingen, die werden gebruikt om de enzymactiviteiten van CYP1A1, 1A2, 2C9 en 3A4 te meten.

Regulatie van CYP1A1, 1A2, 2B6, 2C8, 2D6, 2E1 en 3A4 door de aryl hydrocarbon receptor (AhR), pregnane X receptor (PXR) en constitutieve androstane receptor (CAR) werd bestudeerd in HepG2-cellen op het mRNA- en/of enzymniveau. Regulatie van de CYP1A1-, 1A2-, 2B6- en 3A4-mRNA-niveaus in HepG2 was vergelijkbaar met de regulatie in primaire humane hepatocyten. Echter, CYP2C8-mRNA-niveaus zijn induceerbaar in gecryopreserveerde humane hepatocyten, maar dit werd niet gezien in HepG2-cellen na behandeling met PXR/CAR-activatoren. In overeenstemming met andere studies waren CYP2D6- en 2E1-mRNA-niveaus niet te reguleren met AhR, PXR, en CAR activatoren. De resultaten in hoofdstuk 3 lieten verder zien dat in overeenstemming met up-regulatie mRNA-niveaus ook de enzymactiviteiten van CYP1A1 en 1A2 werden geïnduceerd door AhR agonisten. Vergelijkbaar resulteerde behandeling met PXR-activatoren in een hogere CYP3A4-enzymactiviteit.

Samengevat geven de resultaten in hoofdstuk 3 aan dat de meeste fase I-CYP's aanwezig zijn in HepG2-cellen, maar dan wel op een lager niveau dan in gecryopreserveerde primaire humane hepatocyten. Activatie van de AhR, PXR en CAR leidt tot hogere enzymniveaus en -activiteiten.

Hoofdstuk 4 beschrijft de karakterisatie van het fase II-metabolisme in HepG2-cellen. De mRNA-niveaus van UDP-glucuronosyl transferases (UGTs), sulfotransferases (SULTs), glutathione S-transferases (GSTs), N-acetyltransferase-1 (NAT1) en epoxide hydroxylase-1 (EPHX1), werden gemeten met qPCR in HepG2-cellen en gecryopreserveerde primaire humane hepatocyten en daarna vergeleken. De mRNA-niveaus van SULT1A1, 1A2, 1E1, 1A2, 2A1 en microsomaal GST 1, GST µ1, NAT1 en EPHX1 in HepG2-cellen waren nagenoeg gelijk aan de niveaus in gecryopreserveerde primaire humane hepatocyten. Echter, de niveaus van UGT1A1 en 1A6 waren tussen de 10- en meer dan 1000-voudig lager in HepG2-cellen. De regulatie van de fase II-enzymen door de AhR-, PXR- en CAR-receptoren werd bestudeerd en was vergelijkbaar in HepG2- en primaire humane hepatocyten.

Aangezien fase II-metabolisme kan resulteren in genotoxiciteit kunnen HepG2-cellen een goed modelsysteem zijn om de toxiciteit van zulke teststoffen te bepalen. Voor andere teststoffen zal een combinatie van de lage expressie van CYP's en normale niveaus van fase II-enzymen echter kunnen leiden tot een onderschatting van toxiciteit. Pro-genotoxische testverbindingen die geactiveerd worden door CYP's die laag tot expressie komen, zullen mogelijk moeilijk te detecteren kunnen zijn in HepG2-cellen (bijv. cyclophosphamide die geactiveerd wordt door CYP2B6). Het toepassen van een S9-leverextract of het induceren van CYP-levels in HepG2-cellen door het toevoegen van AhR-, PXR-, of CAR-agonisten zou een oplossing kunnen zijn.

De ontwikkeling van een HCS in vitro micronucleus test in de HepG2- en CHO-k1-cellijn

Om op een snelle manier chromosoomschade (clastogeniciteit en aneugeniciteit) te kunnen vaststellen, zijn twee high content screening (HCS) *in vitro* micronucleus(IVMN)-assays in de humane HepG2-hepatoma-cellijn en hamster CHO-k1-cellijn opgezet. De CHO-k1-cellijn werd gevalideerd omdat deze cellijn dikwijls in de regulatoire gentoxtesten gebruikt wordt.

De genotoxische referentiestof taxol werd gebruikt om de reproduceerbaarheid van beide HCS-IVMN-testen te bepalen. Deze bleek in beide gevallen goed te zijn. Vervolgens werden de testen geëvalueerd met de referentieverbindingen uit de ECVAM-validatielijst. De sensitiviteit (percentage genotoxische carcinogenen die goed voorspeld werden) (80%; 16/20) en specificiteit (percentage niet-genotoxische teststoffen die goed voorspeld werden) (88%; 37/42) van de HCS-IVMN-assay in CHO-k1-cellen was hoog. Opvallend hierbij was dat pro-genotoxische verbindingen die geactiveerd worden door CYP1A-metabolisme, al actief waren in de CHO-k1-cellen zonder de toevoeging van een S9-leverextract. Voorbeelden van zulke stoffen zijn benzo[a]pyrene en 7,12-dimethylbenzanthrene. Dit geeft aan dat CYP1A-metabolisme waarschijnlijk actief was in de gebruikte CHO-k1-cellijn. Toevoeging van een S9-leverextract resulteerde zelfs in een afname van de genotoxische effecten van stoffen die door CYP1A geactiveerd worden.

De sensitiviteit van de HepG2-cellijn was met 60% (12/20) lager dan van de CHO-k1-cellijn. De specificiteit van de HepG2-cellijn had dezelfde hoge waarde als van de CHO-k1-cellijn (88%; 37/42). De intrinsieke metabole activiteit in de HepG2-cellen was hoog genoeg om 7 van de 9 pro-genotoxische testverbindingen in de ECVAM-lijst te activeren: cyclophosphamide, benzo[a]pyrene, 7,12-dimethylbenzanthracene, dimethylnitrosamine, 2,4-diaminotoluene, PhIP.HCl en aflatoxin B1. De twee pro-genotoxische testverbindingen die geen micronuclei inductie in HepG2-cellen gaven waren 2-acetylaminofluorene en IQ. Toevoeging van een S9-leverextract resulteerde voor deze twee teststoffen niet in het genotoxisch worden.

Evaluatie van een additionele set van 16 genotoxische referentieverbindingen bevestigde de resultaten die gevonden werden met ECVAM stoffen lijst. De sensitiviteit van de CHO-k1-cellijn was met 69% (11/16) hoger dan de sensitiviteit van 56% (9/16) die gevonden werd met HepG2-cellijn. Bij beide cellijnen was het mogelijk om de micronuclei op basis van grootte te classificeren, hetgeen een verdere discriminatie van genotoxische teststoffen in aneugene en clastogene teststoffen mogelijk maakt.

Samengevat laten de resultaten in hoofdstuk 5 zien dat twee HCS-IVMN-testen zijn ontwikkeld die op een snelle manier chromosoomschade kunnen detecteren. Met deze testen kunnen genotoxische teststoffen bovendien geclassificeerd worden als clastogeen of aneugeen.

De identificatie van biomarkers voor de bepaling van genotoxiciteit in HepG2-cellen met behulp van genexpressie profilering

De analyse van genexpressieprofielen werd gebruikt om biomarkers voor genotoxiciteit te bepalen. Biomarkers (genen) werden geselecteerd die niet geactiveerd werden door niet-genotoxische levertoxische teststoffen. Zulke biomarkers kunnen gebruikt worden voor het ontwikkelen van high-througput luciferase reporter assays waarmee genotoxische teststoffen gedetecteerd kunnen worden.

HepG2-cellen zijn al eerder gebruikt in genexpressieprofileringstudies waarin de genexpressieprofielen in de HepG2-cellen in staat bleken om een duidelijk onderscheid te kunnen maken tussen genotoxische- en niet-genotoxische carcinogenen. Genen die een discriminerend effect gaven, waren vooral betrokken bij de regulatie van de celcyclus, reparatie van DNA, de immuun- en stressrespons alsook apoptose. Het doel van deze studies was het bepalen van pathways (mechanismen) die betrokken zijn bij genotoxische en niet-genotoxische carcinogeniciteit. Om deze reden werden niet-toxische concentraties van de niet-genotoxische carcinogenen gebruikt.

In het onderzoek dat beschreven is in dit proefschrift werd genexpressieanalyse niet alleen gebruikt voor het bepalen van biomarkers die genotoxiciteit kunnen detecteren, maar de geselecteerde biomarkers moesten bovendien een duidelijk onderscheid kunnen aantonen tussen genotoxische carcinogenen en niet-genotoxische levertoxische teststoffen. Daarvoor werden HepG2-cellen blootgesteld aan cytotoxische concentraties van de niet-genotoxische levertoxische stoffen omdat cytotoxiciteit stresspathways kan activeren en dit mogelijk DNA-schade kan veroorzaken. Het gebruik van cytotoxische stofconcentraties kan daarom leiden tot fout-positieve resultaten voor genotoxiciteit. De verschillen in de genexpressieprofielen van de genotoxische en niet-genotoxisch toxische stoffen werden gebruikt om biomarkergenen of responsieve elementen te bepalen die specifiek waren voor de genotoxische verbindingen. Toepassing van deze biomarkers zou kunnen resulteren in een high-throughput luciferase reporter assay met een goede gevoeligheid voor genotoxiciteit en een laag percentage fout-positieven (voor niet-carcinogenen en in vivo genotoxiciteit).

HepG2-cellen werden gedurende 6, 24 en 72 uur behandeld met 4 genotoxische en 7 niet-genotoxische levertoxische stoffen. Discriminatie tussen de twee stofklasses was beperkt als alle significant gereguleerde genen (DEG's) werden gebruikt. Pathway-analyse van de DEG's in de klasse van genotoxische stoffen liet zien dat meerdere pathways significant gereguleerd werden. De belangrijkste gereguleerde pathways op de drie tijdstippen waren betrokken bij de regulatie van de celcyclus. Op het 72 uur-tijdstip activeerden de niet-genotoxische levertoxische stoffen meerdere pathways die kenmerkend zijn voor genotoxische verbindingen zoals bijv. de p53 pathway, ATM pathway, en G2/M-celcyclus regulatie-pathway.

Om genen te vinden die een duidelijk onderscheid geven tussen de twee klassen van

stoffen werd een meer diepgaande genexpressieanalyse uitgevoerd met behulp van Qlucore Omics Explorer. Deze analyse leverde voor elk tijdstip een cluster (subset) van genen op (6 uur, 362 genen; 24 uur, 1914 genen; 72 uur, 498 genen). Pathway-analyse toonde aan dat de genen in de 6 uur- en 24 uur-gensubsets vooral betrokken waren bij de celcyclus, apoptose en DNA-herstel. Analyse van de 72 uur-subset leverde geen significant gereguleerde pathways op. De genen met de hoogste inductiefactoren (top 10) werden in detail geanalyseerd en bleken voornamelijk downstream targets van p53 te zijn en waren betrokken bij apoptose of de oxidatieve stressrespons. Verder waren er in de drie subsets van genen meerdere genen aanwezig die een belangrijke rol hebben bij de DNA-schadeherstelrespons zoals bijv. GADD45A, GADD45B, PCNA, POLH en XPC.

Samengevat: genexpressieanalyse met Qlucore Omics Explorer resulteerde in drie subsets van genen die een duidelijke discriminatie mogelijk maken tussen genotoxische en niet-genotoxische toxische teststoffen. De genen die de hoogste inductie lieten zien bij de klasse van genotoxische verbindingen, waren voornamelijk betrokken bij regulatie van de celcyclus of waren downstream targets van p53. Het toepassen van deze potentiële biomarkers in luciferase reporter assays zou kunnen resulteren in testsystemen die op een snelle manier genotoxiciteit kunnen detecteren met een laag percentage fout-positieve resultaten.

De ontwikkeling van luciferase reporter assays voor de bepaling van genotoxiciteit en oxidatieve stress

De analyse van genexpressieprofielen in HepG2-cellen liet zien dat de toepassing van promotorgebieden van genen, die betrokken zijn bij de regulatie van de celcyclus of downstream targets van p53 zijn, zou kunnen resulteren in gevoelige luciferase reporter assays voor genotoxiciteit. Deze assays zouden op een snelle manier genotoxiciteit in zoogdiercellen kunnen bepalen met een laag percentage fout-positieve resultaten t.g.v. cytotoxiciteit. De analyse van genexpressieprofielen gaf bovendien aan dat RAD51C een goede biomarker zou kunnen zijn voor stoffen die DNA-dubbelstrengsbreuken veroorzaken. Op basis van de genexpressieprofielen werden de promotorregio's van RAD51C en cystatin A (downstream target van p53, die betrokken is bij apoptose), evenals het responsieve element van het p53-eiwit, geselecteerd voor de ontwikkeling van drie luciferase reporter assays die genotoxiciteit kunnen detecteren. Daarnaast werd een luciferase reporter assay ontwikkeld die de activering van de Nrf2 electrofiele responsieve pathway meet (hoofdstuk 7).

De reproduceerbaarheid van de vier reporter assays werd gemeten met de genotoxische referentieverbindingen doxorubicine en benzo[a]pyrene. De reproduceerbaarheid bleek goed te zijn voor alle vier de testen. Validatie van de drie reporter assays voor genotoxiciteit met de ECVAM-stoffen resulteerde in een voorspelbaarheid (totale

percentage correct voorspelde genotoxische carcinogenen plus niet genotoxische verbindingen) van de individuele reporter assays van 82% voor de HepG2 RAD51C_ luc-assay (sensitiviteit 60%, 12/20; specificiteit 93%, 39/42), van 84% voor de HepG2 cystatin A_luc-assay (sensitiviteit 70%, 14/20; specificiteit 90%, 38/42), en van 90% voor de HepG2 p53_luc-assay (sensitiviteit 85%, 17/20; specificiteit 93%, 39/42). De cumulatieve voorspelbaarheid van de drie HepG2 reporter assays voor genotoxiciteit was hoog met 82% (sensitiviteit 85%, 17/20; specificiteit 81%, 34/42). Het percentage van de genotoxische teststoffen dat de Nrf2 pathway activeerde was hoog met 75% (15/20). Van de niet-genotoxische verbindingen in de ECVAM-lijst activeerde slechts 31% (13/42) de Nrf2 pathway. Verder bleek de metabole capaciteit in HepG2-cellen voldoende te zijn voor activatie van zeven van de negen pro-genotoxische testverbindingen. Deze zeven teststoffen waren benzo[a]pyrene, 7,12-dimethylbenzanthracene, 2-acetylaminofluorene, 2,4-diaminotoluene, IQ, PhIP.HCl en aflatoxin B1. Van deze zeven testverbindingen gaven 2-acetylaminofluorene en IQ een negatief resultaat in de HepG2-HCS-IVMN assay (Hoofdstuk 5). Activatie van het p53 responsive element is daarom een gevoeliger eindpunt voor de detectie van deze twee stoffen dan de vorming van micronuclei (DNA schade). Mogelijk heeft de activatie van de DNA herstelrespons ertoe geleid dat er geen significante inductie van micronuclei in de HepG2-HCS-IVMN-test te meten was. De pro-genotoxische testverbindingen cyclophosphamide en dimethylnitrosamine gaven geen genotoxische respons in de reporter assays. Cyclophosphamide en dimethylnitrosamine worden geactiveerd door respectievelijk CYP2B6 en CYP2E1 (hoofdstuk 3). Deze CYP's komen in HepG2-cellen beide laag tot expressie wat de reden zou kunnen zijn voor het niet genotoxisch worden van deze testverbindingen. Deze hypothese wordt echter niet ondersteund door het feit dat deze twee testverbindingen een positief resultaat geven in de HCS-IVMN-assay (zonder toevoeging van S9-mix) wat aangeeft dat de endogene niveaus van deze enzymen in de HepG2-cellijn blijkbaar hoog genoeg zijn voor metabole activatie van deze twee testverbindingen. Dit geeft verder aan dat voor deze twee testverbindingen de vorming van micronuclei een gevoelig eindpunt is. Opmerkelijk is dat in een studie die uitgevoerd werd door Sohn et al. [3] behandeling van cellen met cyclophosphamide ook geen p53 activatie veroorzaakte.

De resultaten van de drie reporter assays voor genotoxiciteit werden ook vergeleken met de beschikbare data betreffende bacteriële mutageniteit (Amestest), *in vitro* genotoxiciteit in zoogdiercellen en *in vivo* genotoxiciteit voor een extra set van 192 testverbindingen. De voorspelbaarheid voor bacteriële mutageniciteit was 74% (sensitiviteit 61%, 30/49; specificiteit 80%, 77/96), en voor *in vitro* genotoxiciteit in zoogdiercellen 59% (sensitiviteit 45%, 35/78; specificiteit 83%, 38/46). De correlatie tussen de resultaten van de HepG2 reporter assays en *in vivo* genotoxiciteit was hoger met 77% (sensitiviteit 74%, 28/38; specificiteit 81% 26/32). De correlaties van de individuele assays met *in vivo* genotoxiciteit waren 76% voor de HepG2-p53_luc-test

(sensitiviteit 71%, 27/38; specificiteit 81%, 26/32), 59% voor de HepG2-RAD51C_luc-test (sensitiviteit 29%, 11/38; specificiteit 94%, 30/32), en 76% voor de HepG2-cystatin A_luc-test (sensitiviteit 61%, 23/38; specificiteit 94%, 30/32). Zoals ook gemeten werd met de ECVAM-stoffenlijst zijn de HepG2-p53_luc-test en de HepG2-cystatin A_luc-test modelsystemen met een relatief hoge sensitiviteit en specificiteit. De HepG2-RAD51C_luc-test had een lagere gevoeligheid, maar de specificiteit was hoog waaruit blijkt dat slechts een beperkt aantal teststoffen dubbelstrengsbreuken in het DNA veroorzaken. Van de 108 teststoffen in de extra lijst van 192 stoffen die een positief resultaat gaven voor bacteriële of *in vitro/in vivo* genotoxiciteit in zoogdiercellen, activeerde 62% (67 verbindingen) de Nrf2 pathway. Wanneer we binnen deze set van 192 compounds kijken naar de groep van teststoffen zonder of met negatieve resultaten voor genotoxiciteit dan is het percentage Nrf2 positieve stoffen slechts 38% (30/80). Deze resultaten bevestigen de resultaten die gevonden werden met de chemicaliën uit de ECVAM-lijst. Activering van de Nrf2-pathway geeft informatie over het werkingsmechanisme van genotoxische stoffen. Positieve resultaten in de Nrf2-reporter assay kunnen echter niet gebruikt worden voor het identificeren en deselecteren van genotoxische stoffen omdat deze pathway ook wordt geactiveerd door cytotoxische stoffen en diverse stoffen die juist een beschermende werking hebben tegen cytotoxiciteit en genotoxiciteit [4-6]. Deze beschermende werking heeft te maken met het feit dat activering van de Nrf2 pathway resulteert in een verhoogde expressie van fase II- en antioxidant stresseiwitten. Een andere factor die interpretatie van de Nrf2-data moeilijk maakt, is cross-talk tussen de p53- en Nrf2-pathway [7].

Samenvattend kan worden gesteld dat er drie luciferase reporter assays ontwikkeld zijn die gebruikt kunnen worden voor de (snelle) detectie van stoffen met genotoxische eigenschappen. De p53- en cystatin A-reporter assays lieten een hoge sensitiviteit en specificiteit zien voor genotoxische carcinogeniciteit en *in vivo* genotoxiciteit. De RAD51C-reporter assay was veel specifieker en gaf informatie over de vorming van dubbelstrengsbreuken in het DNA. De resultaten in hoofdstuk 7 bevestigden de resultaten die gevonden werden met de analyse van genexpressieprofielen. Er werd ook een Nrf2-reporter assay ontwikkeld die kan helpen bij het ophelderen van het werkingsmechanisme van genotoxische stoffen. Gebaseerd op de resultaten die beschreven staan in hoofdstuk 7 lijkt het dat toepassing van de ontwikkelde high-throughput reporter assays in de vroege discovery fase van het geneesmiddelenonderzoek een heel bruikbare strategie is voor het snel beoordelen van stoffen op genotoxische eigenschappen.

In deze en de vorige paragrafen lag de focus op de ontwikkeling en validatie van testsystemen voor de detectie van teststoffen met genotoxische eigenschappen. Naast de detectie van genotoxische teststoffen zou het ook nuttig zijn om niet-genotoxische carcinogenen vroegtijdig te detecteren in de discovery fase van het geneesmiddelenonderzoek. Niet-genotoxische carcinogenen kunnen tumorvorming

induceren via een groot aantal werkingsmechanismen [8]. Eén van de mechanismen die daarbij een rol speelt is receptor-gemedieerde inductie via de aryl hydrocarbon receptor welke hieronder verder besproken wordt.

De bepaling van CYP1A-inducers en soortspecifieke verschillen tussen de mens en rat

Sterke activering van de AhR kan resulteren in toxische effecten zoals (niet-genotoxische) carcinogeniteit. Aangezien het merendeel van de carcinogeniciteitsstudies wordt uitgevoerd bij ratten, kan de voorspelling van activering van de ratte AhR nuttig zijn. Echter voor de voorspelling van het risico op carcinogeniciteit in de mens zou activering van de humane AhR bestudeerd moeten worden.

Omdat AhR-activatoren een sterke inductie van CYP1A veroorzaken werd CYP1A-inductie in de uitgevoerde studies gebruikt als marker voor AhR-activering. De CYP1A-inductie studies werden uitgevoerd in de humane HepG2- en ratte H4IIE-cellijn. Voor CYP1A-inductie in HepG2- en H4IIE-cellijnen is aangetoond dat deze CYP1A-inductie representatief is voor CYP1A-activering in respectievelijk de mens en rat [2, 9, 10].

Een set bestaande uit 119 teststoffen, waaronder bekende AhR-liganden, werden getest. CYP1A-inductie werd waargenomen voor 24 verbindingen. In H4IIE-cellen, gaven meer teststoffen CYP1A-inductie en de meeste EC50-waarden waren lager dan die in HepG2-cellen. Soortspecifieke CYP1A-inductie in H4IIE- en HepG2-cellen werd waargenomen voor respectievelijk 8 en 3 teststoffen. Aangezien voor meerdere teststoffen soortspecifieke CYP1A-inductie werd gemeten, en het feit dat carcinogeniciteitsstudies in de rat uitgevoerd worden, maar dat de risico's voor de mens ook bepaald moeten worden lijkt parallel screenen in ratte en humane cellen de beste strategie voor de farmaceutische industrie. Teststoffen die CYP1A-inductie laten zien hoeven niet direct gedeselecteerd te worden aangezien lang niet alle CYP1A-inducers dioxineachtige toxiciteit laten zien. Zo zijn er meerdere geneesmiddelen bekend die AhR-activatoren zijn maar geen toxiciteit geven zoals bijv. omeprazol, leflunomide, flutamide en nimodipine [11]. Verder zijn er naast de AhR-pathway nog andere pathways die CYP1A kunnen activeren en daarom leiden tot fout-positieve resultaten. Samenvattend kan worden geconcludeerd dat zowel de ratte- als humane CYP1A-inductieassays nuttig zijn voor het voorscreenen in de discovery fase van de ontwikkeling van geneesmiddelen, maar er moet wel voorzichtig omgegaan worden met het deselecteren van teststoffen die CYP1A inductie geven.

Vergelijking van de sensitiviteit en specificiteit van de regulatoire en high-throughput *in vitro* genotoxiciteitsassays

In dit proefschrift zijn verschillende high-throughput assays voor de detectie van genotoxische stoffen geëvalueerd. In deze paragraaf worden de sensitiviteit en specificiteit van de nieuw ontwikkelde *in vitro* genotoxiciteitstesten met een hoge doorvoersnelheid (high-throughput) vergeleken met de resultaten van de regulatoire genotoxiciteitstesten. Omdat bij de huidige gebruikte regulatoire teststrategie combinaties van assays gebruikt worden om zowel bacteriële mutageniciteit (genmutaties) als genotoxiciteit in zoogdiercellen (chromosoomschade) te kunnen detecteren, zullen ook verschillende combinaties van de high-throughput assays bediscussieerd worden.

Sensitiviteit en specificiteit van gecombineerd gebruik van regulatoire in vitro genotoxiciteitstesten

Het onderscheidende vermogen van individuele en combinaties van de regulatoire *in vitro* genotoxiciteitstesten voor carcinogene en niet-carcinogene stoffen in knaagdieren werd geëvalueerd door Kirkland et al. [12]. Daarbij werd door de auteurs opgemerkt dat het van belang is om de carcinogene stoffen onder te verdelen in genotoxische- en niet-genotoxische carcinogenen, aangezien niet-genotoxische carcinogenen logischerwijs niet gedetecteerd kunnen worden in *in vitro* genotoxiciteitstesten [12]. Genotoxische carcinogenen dienen dus in principe een positief resultaat te geven in *in vitro* genotoxiciteitstesten en niet-genotoxische carcinogenen en niet-carcinogenen een negatief resultaat. Echter voor veel carcinogenen die geëvalueerd werden door Kirkland et al. [12] ontbraken de mechanistische data die een goede classificatie in genotoxische en niet-genotoxische carcinogenen mogelijk maakte. Scores werden daarom berekend t.o.v. carcinogeen/niet-carcinogeen.

De sensitiviteit- en specificiteitscores van de individuele regulatoire genotoxiciteits-assays zijn opgesomd in tabel 3 van hoofdstuk 1. Deze scores laten duidelijk zien dat de specificiteit van de *in vitro* genotoxiciteitstesten in zoogdiercellen voor niet-carcinogenen laag is, wat dus resulteert in een relatief groot aantal fout-positieve resultaten. Normaal gesproken wordt in de regulatoire teststrategie een combinatie van de Amestest + MLA test + chromosoomaberratietest gebruikt (CA) [12] (zie tabel 2). Deze combinatie heeft een hoge sensitiviteit voor knaagdiercarcinogenen van 87,7% (171/202), maar een lage specificiteit voor niet-carcinogenen van slechts 22,9% (22/96). Combinaties van twee regulatoire testen (d.w.z. Ames + CA, AMES + IVMN, AMES + MLA) geven vergelijkbare scores [12]. De overlap tussen de teststoffen die gebruikt werden door Kirkland et al. [12] en de teststoffen die gebruikt werden in dit proefschrift was zeer beperkt. Een directe vergelijking is daarom niet mogelijk.

Voor het berekenen van de sensitiviteit en specificiteit van de nieuw ontwikkelde *in vitro* testsystemen werd in dit proefschrift de ECVAM-stoffenlijst gebruikt [13]. Deze lijst bestaat uit goed gedefinieerde genotoxische carcinogenen, niet-genotoxische carcinogenen en niet-carcinogenen. Deze lijst kan daarom gebruikt worden voor het berekenen van de sensitiviteit en specificiteit voor genotoxische carcinogenen (zie tabel 2). Toepassing van de geaccepteerde regulatoire teststrategie (een combinatie van Ames + MLA + CA-test) geeft voor deze ECVAM-stoffen een sensitiviteit voor genotoxische carcinogenen van 100% (20/20) en specificiteit van 55% (23/42). De lage specificiteit wordt veroorzaakt door de in vitro genotoxiciteitstesten in zoogdiercellen (MLA + CA) die samen 19 fout-positieve resultaten lieten zien in de ECVAM stoffenlijst.

Naast de ECVAM-stoffen is ook een aanvullende set van 192 stoffen gebruikt in dit proefschrift (hoofdstukken 2 en 7). De data betreffende genotoxiciteit en carcinogeniciteit van deze 192 stoffen zijn beperkt en een duidelijke classificatie van deze stoffen in genotoxische carcinogenen en niet-genotoxische stoffen (niet-genotoxische carcinogenen + niet-carcinogenen) ontbreekt. Voor deze stoffen hebben we daarom de keuze gemaakt om de testgegevens te vergelijken met de *in vivo* genotoxiciteitsgegevens, aangezien deze gegevens waarschijnlijk de beste indicatie geven of stoffen genotoxische carcinogenen of niet-genotoxisch zijn. Voor deze aanvullende set van 192 stoffen geeft de combinatie AMES + *in vitro* genotoxiciteitstesten in zoogdiercellen (zie tabel 2) een sensitiviteit van 97% (37/38) en specificiteit van 41% (12/29). Ook hier werd de lage specificiteit veroorzaakt door de *in vitro* genotoxiciteitstesten in zoogdiercellen (zie tabel 2).

Samenvattend kan gesteld worden dat, ondanks dat de manier van vergelijken verschillend is (verschillende eindpunten zie tabel 2) de resultaten met de twee stoffensets die gebruikt werden in het huidige proefschrift, vergelijkbare scores voor de regulatoire testen geven als de scores die verkregen werden met de studie die beschreven werd door Kirkland et al. [12]. Een combinatie van de AMES + *in vitro* genotoxiciteitstesten in zoogdiercellen heeft een hoge gevoeligheid, maar lage specificiteit. De ECVAM-stoffenlijst en additionele set van 192 compounds maken een direct vergelijk tussen de resultaten van de regulatoire genotoxiciteitstesten (Ames + *in vitro* assays zoogdieren) en de in dit proefschrift ontwikkelde high-throughput assays mogelijk. Verschillende combinaties van de in dit proefschrift ontwikkelde high-throughput assays worden in de volgende paragraaf besproken en de scores zullen worden vergeleken met die van de regulatoire *in vitro* genotoxiciteitstesten.

Sensitiviteit en specificiteit van gecombineerd gebruik van de high-throughput genotoxiciteitstesten en vergelijking met de regulatoire in vitro genotoxiciteitstesten

Door als uitgangspunt te nemen dat een vroege screen voor genotoxiciteit bacteriële mutageniciteit moet kunnen detecteren (genmutaties) moet in een combinatie van high-throughput assays in ieder geval de Vitotox™-test aanwezig zijn. De hoge sensitiviteit

en specificiteit van de Vitotox™ voor Amesresultaten van respectievelijk meer dan 80% en 90% (hoofdstuk 2), de hoge throughput en kleine stofhoeveelheid die nodig is, zijn eigenschappen die dit testsysteem een waardevolle prescreen voor Amesresultaten maken. De Vitotox™-test kan dus op een snelle manier Amesresultaten goed voorspellen in de vroege discovery fase van het geneesmiddelenonderzoek. Naast bacteriële mutagenen moet een vroege screen voor genotoxiciteit ook teststoffen detecteren die chromosoomschade veroorzaken. In dit proefschrift werden meerdere assays geëvalueerd die de potentie hebben om chromosoomschade te detecteren: (1) de op gistcellen gebaseerde RadarScreen-test, (2) de CHO-k1-HCS-IVMN-assay, (3) de HepG2-HCS-IVMN-assay, en (4) de HepG2 p53-luciferase reporter assay. Combinaties van deze 4 testsystemen met de Vitotox™-assay worden hieronder besproken. De sensitiviteit en specificiteit scores van het gecombineerde gebruik zijn samengevat in tabel 3.

1. Vitotox + RadarScreen-test

Het testen van de ECVAM-stoffen resulteerde in een hoge sensitiviteit (80%) en specificiteit (81%). Daarentegen was de specificiteit die gemeten werd met de aanvullende set van stoffen laag met slechts 28%. Deze combinatie van testen is daarom moeilijk te gebruiken in de vroege discovery fase van het geneesmiddelenonderzoek. Bij gebruik van deze combinatie en het deselecteren van stoffen die een positief resultaat geven, zouden wellicht te veel stoffen gedeselecteerd worden.

2. Vitotox + CHO-K1-HCS-IVMN-assay

Deze combinatie geeft voor de 62 ECVAM-stoffen een sensitiviteit van 95% (19/20). De specificiteit van de combinatie Vitotox™ + CHO-K1 HCS IVMN is met 83% (35/42) veel hoger dan de specificiteit van 55% (23/42) die de combinatie van de regulatoire Ames- + *in vitro* genotoxiciteitstesten in zoogdiercellen geeft. Deze scores geven aan dat gecombineerd gebruik van de Vitotox™ + CHO-K1-HCS-IVMN-test waardevol kan zijn voor het vroeg screenen op genotoxische eigenschappen van stoffen.

Wel is het zo dat additionele validatie aan te bevelen is aangezien de regulatoire IVMN-test in CHO-k1-cellen een veel lagere specificiteit heeft (30,8% voor niet-carcinogenen [12]). Redenen voor deze opmerkelijke grote verschillen tussen eerdere en recente studies beschreven in dit proefschrift, kan de aanwezigheid van een functioneel actief p53-eiwit in de gebruikte CHO-k1-cellijn zijn. Uit niet gepubliceerde studies met de p53-remmer pifithrin-α blijkt dat p53 op zijn minst deels functioneel is in de CHO-k1-cellijn die gebruikt werd in dit proefschrift. Een andere reden voor het grote verschil in specificiteit zou de toepassing van de HCS-techniek kunnen zijn aangezien de variatie die normaal gesproken aanwezig is bij het visueel tellen van micronuclei in de regulatoire IVMN-test afwezig is bij gebruik van de HCS-techniek [14].

Tabel 2. Sensitiviteit and specificiteit bij gecombineerd gebruik van de regulatoire in vitro genotoxiciteits testen voor drie sets van teststoffen.

Stoffenset	**Kirkland et al. [12]**	**Kirkland et al. [13] (ECVAM)**	**Dit proefschrift (extra teststoffenset)**		
Eindpunt	**Carcinogeniciteit**	**Genotoxische carcinogeniciteit**	**In vivo genotoxiciteit**		
Testen	1. Ames 2. in vitro gentox assays in zoogdiercellen	1. Ames 2. in vitro gentox assays in zoogdiercellen	1. Ames	1. in vitro gentox assays in zoogdiercellen	1. Ames 2. in vitro gentox assays in zoogdiercellen
Sensitiviteit %	84,7 (171/202)	100 (20/20)	48 (16/33)	94 (34/36)	97 (37/38)
Specificiteit %	22,9 (22/96)	55 (23/42)	79 (22/28)	46 (12/26)	41 (12/29)

Tabel 3. Sensitiviteit en specificiteit scores voor verschillende combinaties van de high-throughput genotoxiciteits modelsystemen die in dit proefschrift geëvalueerd werden. De scores zijn weergegeven voor de extra stoffenset en/of ECVAM stoffen lijst.

Test combinatie	**1. Vitotox™ 2. RadarScreen**		**1. Vitotox™ 2. CHO-k1 IVMN**	**1. Vitotox™ 2. HepG2 IVMN**	**1. Vitotox™ 2. HepG2 p53_luc**	
Naam Stoffenset	ECVAM	Extra	ECVAM	ECVAM	ECVAM	Extra
Sensitiviteit (%)	80 (17/20)	73 (27/37)	95 (19/20)	90 (18/20)	85 (17/20)	76 (29/38)
Specificiteit (%)	81 (34/42)	28 (9/32)	83 (35/42)	83 (35/42)	88 (37/42)	72 (23/32)

3. Vitotox + HepG2-HCS-IVMN-assay

Voor de ECVAM-teststoffen geeft een combinatie van deze twee testen een sensitiviteit van 90% (18/20) en een specificiteit van 83% (35/42). Net als al gemeten werd voor Vitotox™ + CHO-k1-HCS-IVMN-combinatie is de specificiteit van de HCS-assays veel hoger dan de specificiteit van de Ames + *in vitro* genotoxiciteitstesten in zoogdiercellen. De sensitiviteit- en specificiteitscores geven aan dat gecombineerd gebruik van de Vitotox™ + HepG2-HCS-IVMN-test een goede strategie zou kunnen zijn voor het screenen in de discovery fase.

4. Vitotox + HepG2-p53_luc-test

Deze combinatie van testsystemen geeft voor de ECVAM-teststoffen een gevoeligheid van 85% (17/20) en een specificiteit van 88% (37/42). Slechts drie genotoxische carcinogenen worden niet gedetecteerd en de specificiteit van 88% is veel hoger dan de specificiteit van 55% (23/42) met de regulatoire testcombinatie Ames- + *in vitro* genotoxiciteitstesten in zoogdiercellen. Voor de extra set van teststoffen zijn de sensitiviteit en specificiteit respectievelijk 76% (29/38) en 72% (23/32). Deze sensitiviteit is lager dan van de combinatie Ames- + *in vitro* genotoxiciteitstesten in zoogdiercellen (97%, 37/38). De specificiteit is met 72% veel hoger dan de 41% (12/29) die gemeten wordt bij gecombineerd gebruik van de Ames- + *in vitro* genotoxiciteitstesten in zoogdiercellen. Evaluatie met de twee sets van stoffen laat dus duidelijk zien dat gecombineerd gebruik van de Vitotox™-test + HepG2-p53_luc-test een hoge sensitiviteit en specificiteit geeft. Dit maakt dat deze combinatie nuttig zou kunnen zijn voor het snel screenen op genotoxiciteit in de discovery fase van de geneesmiddelenontwikkeling. Een voordeel van deze combinatie is dat de validatie werd uitgevoerd met een grote set van teststoffen (ECVAM + extra set van 192 stoffen).

Combinaties van de Vitotox™ met de cystatin A- en RAD51C-reporter assays zullen hier niet worden besproken omdat zowel cystatin A als RAD51C voor hun transcriptie afhankelijk zijn van p53-activatie [15]. Het gebruik van deze testen geeft daarom geen betere scores (hoofdstuk 7). Dus van deze drie reporter assays, blijkt de p53-reportertest het meest waardevol te zijn voor het detecteren van stoffen met genotoxische eigenschappen. Het uitvoeren van de aanvullende assays zoals de cystatin A-, RAD51C-, maar ook Nrf2-reporter-assays kan wel van nut zijn aangezien deze testsystemen informatie geven over het mechanisme van genotoxische stoffen. Het gebruik resulteert echter niet in het detecteren van meer genotoxische stoffen (of in een daling van de specificiteit). Zoals al eerder besproken in deze samenvatting kan de Nrf2- reporter nuttig zijn maar kan deze assay niet gebruikt worden voor het deselecteren van compounds.

Samenvattend kan gesteld worden dat er vier combinaties van high-throughput genotoxiciteitstesten zijn geëvalueerd. De combinatie Vitotox™ + RadarScreen heeft een specificiteit die te laag is. De andere drie combinaties (Vitotox™ + CHO-K1-HCS-IVMN-assay, Vitotox™ + HepG2-HCS-IVMN-assay en Vitotox™ + HepG2 p53_luc)

geven goede scores voor de sensitiviteit en specificiteit wat aangeeft dat gebruik van deze combinaties voor screening in de vroege discovery fase nuttig kan zijn. De specificiteit van deze drie combinaties is hoger dan van gecombineerd gebruik van de regulatoire testcombinatie Ames + *in vitro* genotoxiciteitstesten in zoogdiercellen. Van de drie combinaties is de Vitotox™- + HepG2-p53_luc-combinatie degene die is gevalideerd met het grootste aantal teststoffen wat het meeste vertrouwen in deze combinatie geeft. Daarom is gebruik van deze combinatie (in eerste instantie) aan te bevelen om als teststrategie te gebruiken in de vroege fase (discovery) van het geneesmiddelen onderzoek.

Verdere validatie van de HCS-IVMN-testen met meer teststoffen wordt tevens aanbevolen. Afhankelijk van de resultaten kan een HCS-IVMN-assay worden toegevoegd aan de Vitotox™- + p53_luc-combinatie. Dit zal een directe discriminatie van genotoxische stoffen in clastogene en aneugene stoffen mogelijk maken.

Conclusie

In dit proefschrift zijn meerdere high-throughput testsystemen voor het detecteren van stoffen met genotoxische en niet-genotoxische carcinogene eigenschappen ontwikkeld en gevalideerd met referentieverbindingen. De resultaten die beschreven zijn in dit proefschrift laten zien dat een vroegtijdige voorspelling gedaan kan worden voor bacteriële mutageniteit (genmutaties), chromosoomschade en activering van de AhR.

Verschillende combinaties van de high-throughput testsystemen werden geëvalueerd om de beste strategie te bepalen voor het vroeg in de discovery fase kunnen detecteren van genotoxiciteit. De combinatie Vitotox™- + HepG2-p53_luc-reporter-assay is gebaseerd op de gepresenteerde resultaten in dit proefschrift het meest bruikbaar voor het screenen van teststoffen op genotoxische eigenschappen. Deze combinatie geeft een hoge sensitiviteit en een laag risico op fout-positieve resultaten. Parallel aan de Vitotox™-assay en HepG2-p53_luc-reporter-assay kunnen CYP1A-inductieproeven in menselijke HepG2- en ratte-H4IIE-cellen uitgevoerd worden om mogelijke niet-genotoxische carcinogene AhR-activatoren te detecteren. Toepassing van deze testsystemen zou waardevol kunnen zijn in toekomstige strategieën voor de ontwikkeling van geneesmiddelen.

Additionele opmerkingen

De regulatoire genotoxiciteitstesten en de genotoxiciteitstesten in de discovery fase worden in het algemeen serieel uitgevoerd. Voor elke individuele assay wordt teststof besteld en vervolgens worden verdunningsreeksen gemaakt. Deze manier van werken is relatief arbeidsintensief en resulteert bovendien in verbruik van grote hoeveelheden teststof. Dit is vooral in de vroege discovery fase een probleem omdat het aantal stoffen dat getest moet worden in die fase hoog is en bovendien is de hoeveelheid stof die beschikbaar is klein. Een strategie waarin testen parallel uitgevoerd worden zou een efficiente alternatieve strategie kunnen zijn in de discovery fase [16]. Bij parallel screenen wordt maar één keer stof besteld en een verdunningsreeks gemaakt. Deze verdunningen worden vervolgens (m.b.v. een pipetteerrobot) toegevoegd aan assayplaten van verschillende testen die parallel uitgevoerd worden. Deze strategie resulteert in een effectief gebruik van de kleine beschikbare hoeveelheid stof en het snel beschikbaar komen van (veel) assaydata.

Een andere belangrijke determinant voor het succes van het vroeg screenen is de zuiverheid van de teststoffen. In de vroege fase van het geneesmiddelenonderzoek is er een bepaald evenwicht tussen de snelle synthese van nieuwe verbindingen en het percentage onzuiverheden in batches van de teststoffen. In testen die de farmacologische eigenschappen van deze stoffen bepalen, zijn kleine hoeveelheden verontreinigingen vaak geen probleem omdat lage concentraties teststof over het algemeen voldoende zijn voor farmacologische activiteit. Daarentegen moeten voor de analyse van genotoxische eigenschappen hoge concentraties tot zelfs 10^{-3} M getest worden. Door deze hoge concentraties kunnen verontreinigingen die maar in kleine hoeveelheden aanwezig zijn, toch fout-positieve resultaten veroorzaken. De zuiverheid van de stoffen in de discovery fase moet daarom hoog genoeg zijn om niet te veel fout-positieve resultaten te veroorzaken. Per geval kan voor farmacologisch interessante verbindingen die een positief resultaat in de genotoxiciteitstesten geven, besloten worden om de stoffen opnieuw te testen na opzuivering met HPLC of kristallisatie.

De toepassing van een S9-metabool systeem in HepG2- en CHO-k1-cellen verdient ook een opmerking. Ondanks dat wijzelf (hoofdstuk 5) en anderen [17] hebben aangetoond dat het mogelijk is om een S9-metabool systeem in combinatie met HepG2- en CHO-k1-cellen te gebruiken om pro-genotoxische teststoffen zoals aflatoxin B1 te activeren, laten de resultaten in dit proefschrift ook duidelijk zien dat het gebruik van een S9-metabool systeem soms verschillende resultaten geeft in modelsystemen in bacteriën en gistcellen t.o.v. CHO-k1- en HepG2-cellen. In bacteriën en gistcellen waren pro-genotoxische stoffen niet actief zonder de additie van S9-mix. Na de additie van S9-mix werden de pro-genotoxische teststoffen geactiveerd (positief resultaat

voor genotoxiciteit). Ditzelfde werd waargenomen voor pro-genotoxische teststoffen in CHO-k1- en HepG2-cellen wanneer endogeen metabolisme, dat nodig was voor activatie van deze testverbindingen, niet of zeer beperkt aanwezig was in de cellijnen. Wanneer er echter al een sterke activatie van de pro-genotoxische teststoffen werd waargenomen (door endogeen metabolisme) zonder toevoeging van S9-mix, resulteerde toevoeging van S9-mix juist in een afname van de genotoxische effecten. De bacteriën en gistcellen die in dit proefschrift gebruikt zijn, bevatten genetische modificaties waardoor de celmembranen beter doorlaatbaar worden voor teststoffen zodat deze het DNA makkelijk kunnen bereiken. Bovendien hoeven de teststoffen in bacteriën slechts één membraan te passeren om het DNA te bereiken. In de HepG2- en CHO-k1-cellijn kunnen de hydrofiele metabolieten die gevormd worden door de S9-mix buiten de cel waarschijnlijk het DNA minder makkelijk bereiken dan de teststof zelf. Dit zou kunnen resulteren in een kleinere hoeveelheid reactieve metaboliet in de cellen dan in de situatie waarin de teststof geactiveerd wordt door endogeen metabolisme.

Een ander opmerking die hier geplaatst wordt gaat over de methode van validatie. De validaties die uitgevoerd werden in dit proefschrift geven een eerste goede indicatie over de bruikbaarheid en reproduceerbaarheid van de testen. De validaties werden echter niet uitgevoerd volgens de officiële ECVAM-richtlijnen. Een validatie volgens ECVAM-richtlijnen is echter wel noodzakelijk om te komen tot acceptatie van een test door regulatoire autoriteiten. De validatie volgens de ECVAM-procedure is een langdurig proces dat bestaat uit een groot aantal stappen (http://tsar.jcr.ec.europa.eu/). De kracht van de in dit proefschrift ontwikkelde testsystemen is de praktische toepasbaarheid in de vroege fase van de ontwikkeling van geneesmiddelen. Voor deze toepassing is een officiële validatie volgens de ECVAM-regels niet nodig. Echter, voor een aantal van de ontwikkelde testen zoals bijv. de p53-luciferase reporter assay zou regulatoire erkenning uiteindelijk wel nuttig kunnen zijn.

De laatste opmerking gaat over modelsystemen die in de toekomst ontwikkeld zouden kunnen worden. De analyse van genexpressieprofielen met genotoxische- en niet-genotoxische toxische teststoffen resulteerde in meerdere biomarkers die gebruikt kunnen worden voor de discriminatie tussen genotoxische en niet-genotoxische teststoffen (die een cytotoxische werking hebben). Gebaseerd op de genexpressieprofielen werden drie luciferase reporter assays ontwikkeld. Twee van deze assays bleken geschikt te zijn voor het detecteren van teststoffen met genotoxische eigenschappen (p53- en cystatin A-reporters). De RAD51C-reporter assay was specifieker en identificeerde een bepaald werkingsmechanisme van de genotoxische verbindingen, namelijk de vorming van DNA-dubbelstrengsbreuken. Aanvullende genexpressieprofileringsstudies in HepG2-cellen zouden nuttig zijn voor het bepalen van additionele specifieke biomarkers die ook andere werkingsmechanismen van genotoxische teststoffen zouden kunnen ophelderen. Zo zou het nuttig zijn om een set te hebben van biomarkers die een onderscheid kunnen

maken tussen direct werkende genotoxische stoffen, genotoxische stoffen die DNA cross-linking veroorzaken, topoisomerase remmers, DNA-synthese remmers, stoffen die oxidatieve stress veroorzaken, en aneugenen. Dergelijke biomarkergenen kunnen dan weer gebruikt worden voor de ontwikkeling van high-throughput testsystemen zoals luciferase reporter assays. Zoals eerder besproken in dit proefschrift kunnen high-throughput assays die niet-genotoxische carcinogenen detecteren, nuttig zijn in de discovery fase van het geneesmiddelenonderzoek. In dit proefschrift zijn twee van zulke testen geëvalueerd. Deze twee testsystemen werden gebruikt om de activatie van de humane- en ratte-AhR-receptor te meten. Activatie van de AhR is echter slechts één van de vele mechanismen die niet-genotoxische carcinogeniteit veroorzaakt. Activering van een aantal andere receptoren dan de AhR kan ook resulteren in een niet-genotoxische carcinogeniteit. Voor dergelijke receptoren, zoals de peroxisome proliferator activated receptor α, CAR en thyroid hormoonreceptor kan het daarom wellicht ook nuttig zijn om screeningstesten te ontwikkelen in humane- en/of knaagdiercellijnen.

Samenvattend kan gesteld worden dat er in dit proefschrift nieuwe snelle genotoxiciteitstesten ontwikkeld zijn. Evaluatie heeft aangetoond dat een aantal van deze testen een betrouwbaar high-throughput alternatief zijn voor de regulatoire genotoxiciteitstesten. Vooral bepaalde combinaties van de ontwikkelde testen blijken uitstekende mogelijkheden te bieden voor het snel screenen van teststoffen op genotoxische eigenschappen zonder het risico op veel fout-positieve resultaten. Bovendien zijn twee testsystemen ontwikkeld voor het detecteren van AhR-activatoren in de mens en rat. Een aantal van de nieuw ontwikkelde testen in dit proefschrift zal daarom zijn nut kunnen bewijzen in toekomstige teststrategieën voor de ontwikkeling van geneesmiddelen.

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Dankwoord, Curriculum vitae, List of publications, Overview of completed training activities

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Curriculum vitae

Walter Westerink was born in Almelo on 7 January 1975. He finished his secondary school at the Pius X College (VWO) in 1993. The same year he started the Bsc study Medical Biochemistry at the Hogeschool Enschede. In July 1997 he graduated (with distinction) and became research assistant at NV Organon were he studied the effects of estrogens on the cardiovascular system and bone. In 2000 he became as senior research assistant involved in the development and implementation of assays for early toxicity screening in the discovery phase of drug development. During this period he got inspired for high-throughput screening, molecular toxicology and genotoxicity. In 2005 he started the study molecular life sciences at the HAN University (Nijmegen). The main topics were infection diseases, biotechnology and carcinogenesis. In February 2007 he acquired his Master of Science degree ‘with distinction’. From 2006 he carried out the investigations described in this thesis. Meanwhile NV Organon was acquired by Schering-Plough and thereafter Schering-Plough merged with MSD. Presently, he is working at MSD as scientist in the department of toxicology and drug disposition where he is involved in mechanistic and investigative toxicology.

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Manuscripts submitted or in preparation

W.M. Westerink, T.J. Schirris, G.J. Horbach, W.G. Schoonen. Development and validation of a high content screening in vitro micronucleus assay in CHO-k1 and HepG2 cells, submitted.

W.M. Westerink, A.A. van Staalduinen, J. Polman, W.G. Schoonen, J.P. Groten. The identification of biomarkers for genotoxicity in HepG2 cells by gene expression profiling, in preparation.

Overview of completed training activities

Courses

Laboratory animal science catch 9 (PET)	2000
Molecular toxicology (PET)	2007
Organ toxicology (PET)	2007
Mutagenesis/carcinogenesis (PET)	2008
Reproductive toxicology (PET)	2008
Toxicological risk assessment (PET)	2009

Meetings

PhD symposium (NVT)	2005
Invitox 2006 (ESTIV, VITO)	2006
Eurotox 2007 (ESTIV)	2007
PhD symposium (NVT)	2008
PhD symposium (NVT)	2009
BELTOX/INVITROM meeting	2009

General courses

Radiation expert 5B (TU Delft)	2000
Technisch wetenschappelijk rapporteren (AKZO Nobel)	2000
Laboratory animal science catch 12 (Hogeschool Utrecht)	2000
QPCR Basic training (Applied Biosystems)	2000
Reactiekinetiek (Hogeschool Utrecht)	2001
English language 2 (NIOW)	2003
Good laboratory practice course (MSD)	2010

Optionals

Preparation of PhD research proposal
MSc course General Toxicology WUR-TOX 30202
Thesis and research in progress presentations, Organon/Schering-Plough/MSD, 2006-2010

PET = Postdoctoral Education in Toxicology
NVT = Netherlands Society of Toxicology
ESTIV = European Society of Toxicology In Vitro
VITO = Flemish Institute for Technological Research
MSD = Merck Sharp & Dohme

Approved by the graduate school VLAG

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