Oxidative stress responses in hepatocarcinogenesis: unravelling the mechanisms using a toxicogenomics approach

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Oxidative stress responses in hepatocarcinogenesis:

Unravelling the mechanisms using a toxicogenomics approach

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CHAPTER 1

General Introduction

Biology of Hepatocellular carcinoma

Cancer worldwide

Each year, cancer strikes millions of people worldwide, accounting for 1 out of 4 deaths and therefore being one of the leading causes of death [1]. Together with the millions of estimated new cases of cancer, each year cancer inflict an enormous burden of human suffering, but also high economic costs [2]. This can partly be avoided by investing in prevention or early detection. Obtaining more insights in molecular mechanisms and early treatment of this disease has therefore been a main concern of researchers worldwide. This has already led to a significant progress in knowledge in the field of prevention by defining risk factors for cancer, however, insights in molecular mechanisms of these risk factors in the formation of cancer are still fragmentary and unclear. Therefore, cancer stays a growing cause of death worldwide [1].

Hallmarks of cancer

The human body consists out of 10^{15} cells which divide and differentiate in a strictly controlled way by a complex network of molecular mechanisms. These mechanisms can program each cell to proliferate or on the other hand to induce apoptosis. Each factor or mutation that disturbs this controlled balance has a potential to change, if not corrected, the total number of cells in a specific organ or tissue, which may initiate uncontrolled cell growth. However, several alterations in the cells are required for the progressive conversion of normal cells to cancer cells (Figure 1). The vast majority of mutations that give rise to cancer are not inherited, but are mainly induced spontaneously by chemical damage to regions in DNA that are important for normal cellular function [3]. Moreover, 90-95% of cancer incidences are induced and promoted by environmental factors such as radiation, drugs, diet, pollution, infections, stress and our own life style [4, 5]. Extreme variation in cancer incidence between different tissues exist which correlates with the total number of cell divisions to maintain tissue's homeostasis [6]. This means that rapid dividing cells are more susceptible for mutations induced by such environmental factors.

Mutations can occur in oncogenes, leading to an increased activity of their translated proteins that positively regulate cell proliferation. On the other hand, mutations can also occur in tumor suppressor genes which will lead to inactivation of proteins that negatively regulate cell proliferation [3]. Occurrences of these types of mutations are considered to be the initial event in carcinogenesis. This is followed by different alterations on cellular level that provides the cells with growth advantages by enabling independent proliferative signaling. Production and release of growth-promoting and –inhibiting signals are carefully controlled in normal tissue to guide the cells into cell division to maintain normal tissue function and cell number. Cancer cells on the contrary, deregulate these signals to sustain proliferative signaling by producing growth hormones themselves or by stimulating

normal cells to supply the cancer cells with various growth signals [7, 8]. Moreover, cancer cells can evade growth suppression signals by inactivation of tumor suppressor genes by genetic or epigenetic changes [9]. In addition, they possess different strategies to circumvent apoptosis such as inactivation of the tumor suppressor TP53 or upregulation of antiapoptotic regulators such as Bcl-2 and downregulation of proapoptotic regulators such as Bax [9].

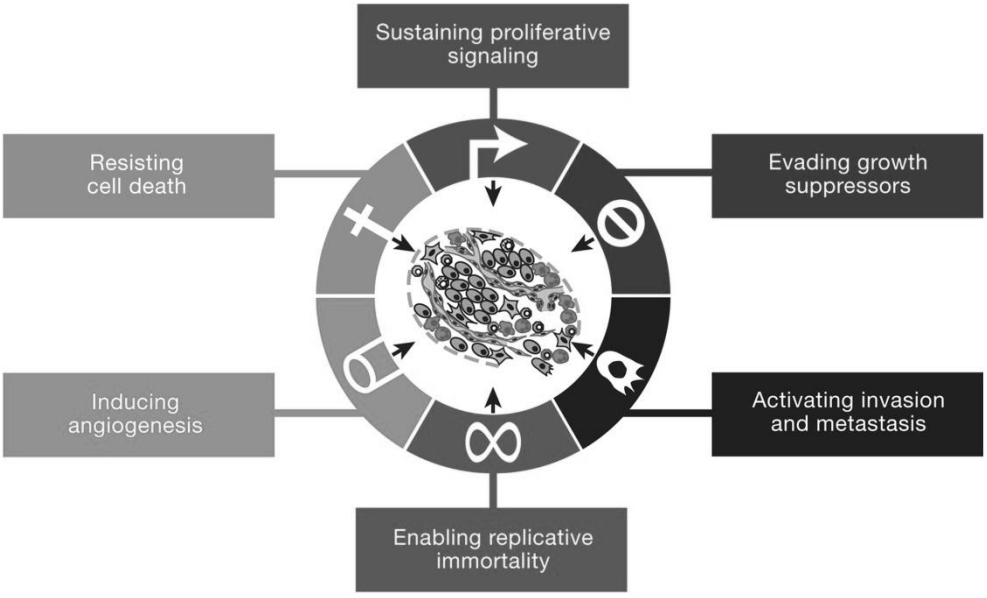

Figure 1 The hallmarks of cancer. Obtained from Hanahan and Weinberg [9]

As a result of these obtained abilities, cancer cells are capable to replicate unlimitedly to generate macroscopic tumors. This tumor promotion is characterized by gene expression alterations and requires non-mutagenic stimuli that disrupt tissue and induce inflammation which results in an enhanced proliferation of cancer cells. Angiogenesis is activated during this step to provide cancer cells with nutrients and oxygen to help sustain expanding neoplastic growths [10]. In this way, the tumor transitions to limitless, invasive and metastatic growth, characterized by changes in number and/or arrangements of chromosomes, as well as the loss of E-cadherin, an inhibitor of invasion and metastasis [11].

Continuous exposure to toxic environmental factors may induce and promote this process and therefore presents an important factor that contributes to the development of various cancers such as hepatocellular carcinoma as described in the following paragraph.

Hepatocarcinogenesis

Hepatocellular carcinoma (HCC) is one of the most malignant and frequent cancers in the world [12]. HCC is mostly diagnosed at an advanced stage because

the lack of early biomarkers. Therefore, surgery and liver transplantation are mostly not successful due to high recurrence and metastasis [13]. Moreover, because of high intrinsic drug resistance, treatment with chemotherapy is not a great success for most patients [14, 15]. As a result, HCC is the third most common cause of cancer deaths worldwide [12, 16]. Major risk factors are persistent infections with Hepatitis B and C viruses (80%) [13, 17]. Other risk factors include, alcohol, obesity and carcinogenic compounds present in diet, environment and drugs [13, 18].

Features of the liver in relation to HCC

This frequent occurrence of HCC is mainly due to the unique vascular and metabolic features of the liver. The liver is the major detoxification organ in the human body and is therefore one of the primary organs prone to damage induced by environmental factors. Portal blood brings compounds absorbed by the gut directly to the liver in a concentrated form [19]. Cytochrome p450 enzymes present in the liver can detoxify many xenobiotics, however, they can also activate toxicity by creating toxic metabolites.

In normal situations, the liver exhibits low levels of cell division, however, during toxic events, massive proliferation can be induced for tissue regeneration [3]. This active proliferative response is an important target for carcinogenic factors [19]. As previously described, there is growing evidence that also HCC develops by the accumulation of mutations and (epi-)genetic alterations leading to malignant transformations [20, 21]. In general, though, histopathological and molecular features that lead to HCC initiation and progression are still poorly understood [22].

The normal liver lobule consists out of hepatocytes, cholangiocytes and nonparenchymal cells such as Kupffer, sinusoidal endothelial and stellate cells while natural killer cells (NK cells) and lymphocytes are located in the sinusoidal lumen of the liver [23]. During toxic challenges, Kupffer cells as well as neutrophils are activated and can contribute to liver toxicity by inducing reactive oxygen species which cause oxidative stress. As a result, liver cirrhosis may occur, the most advanced stage of fibrosis which is characterized by the disruption of the liver parenchyma. The process of recurrent liver cell necrosis and regeneration with increased cell turnover sensitizes liver cells to the adverse effects of other mutagenic agents [23]. Both genetic and epigenetic changes may occur in this event, ultimately leading to the formation of dysplastic foci, nodules, and finally HCC [23, 24].

Gene expression in HCC

Differences in gene expression in the formation of HCC happen as a multistep process which involves a number of factors including genetic alterations formed by point mutations, abnormal expression of proteins, inhibition of tumor suppressors, activation of oncogenes, and molecular DNA adducts that regulate these events, such as microRNA, DNA methylation (paragraph p16) or cellular proteins [13]. Multiple studies have shown that a number of critical signaling

pathways are deregulated in HCC, such as Wnt/β-catenin signaling [25]. This pathway regulates homeostasis, cell proliferation, differentiation, motility and apoptosis, and alterations of this pathway are thought to represent an early oncogenic event in HCC. Deregulation of this pathway may also alter the expression of glutathione S-transferase, as observed in human HCC [26]. In addition, tumor suppressor genes p53, Rb1, CDKN2A, IGF2R and PTEN are inactivated in HCC patients as well as activation of oncogenes β-catenin, Axin1, PI-3-kinase and K-ras [13]. Gain-of-function mutations in β-catenin are common alterations in HCC and lead to activation of nuclear factor-kappa B (NF-κB), a regulator of inflammation and cell death in the development of hepatotoxicity and hepatocarcinogenesis [27]. On the other hand, p53 inactivation is associated with shorter survival of HCC patients due to the loss of cell cycle arrest and apoptotic signals [28]. Also, different studies have shown that mitogen-activated protein kinases (MAPK) play a role in HCC by promoting cell proliferation [29]. In addition, cancer-associated fibroblasts are found to be important in HCC initiation and promotion by producing epidermal growth factor (EGF), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), interleukin 6 (IL-6), chemokine ligand 12 (CXCL12) and matrix metalloproteases 3 and 9 (MMP-3 and MMP-9).

Different risk factors of HCC are capable of targeting these signaling pathways and genes. Although these inducers of HCC have different modes of action, their common feature seems to be the formation of reactive oxygen species (ROS) [30, 31].

Oxidative stress

Damage induced by free radicals and reactive oxygen species

Every cell contains a well-balanced reducing redox environment to maintain homeostasis, controlled by different enzymes. Any imbalance in the normal redox state may result in an increased cellular production of free radicals and may evolve in oxidative stress [23]. Free radicals play a role in a diverse array of biological processes such as normal cell growth, immune responses, cell death and cellular senescence [32]. For example, ROS such as superoxide ($O_2^{\bullet -}$) or hydroxyl radical ($HO^{\bullet}$), are continuously produced endogenously by all aerobic cells as byproducts of various metabolic reactions. Major sources of ROS are the mitochondrial respiratory chain and activated phagocytic cells which increase the activity of NADPH oxidase in order to protect the host against pathogens [32, 33]. Additionally, in the liver, the different isoforms of cytochrome P450 prevent acute toxic effects from chemicals but also result in oxidant byproducts [34, 35]. ROS can be eliminated by protective mechanisms, referred to as antioxidants. Under normal physiological conditions, a balance exists between oxidants and antioxidants (Figure 2). A shift in this balance determines the extent of oxidative stress.

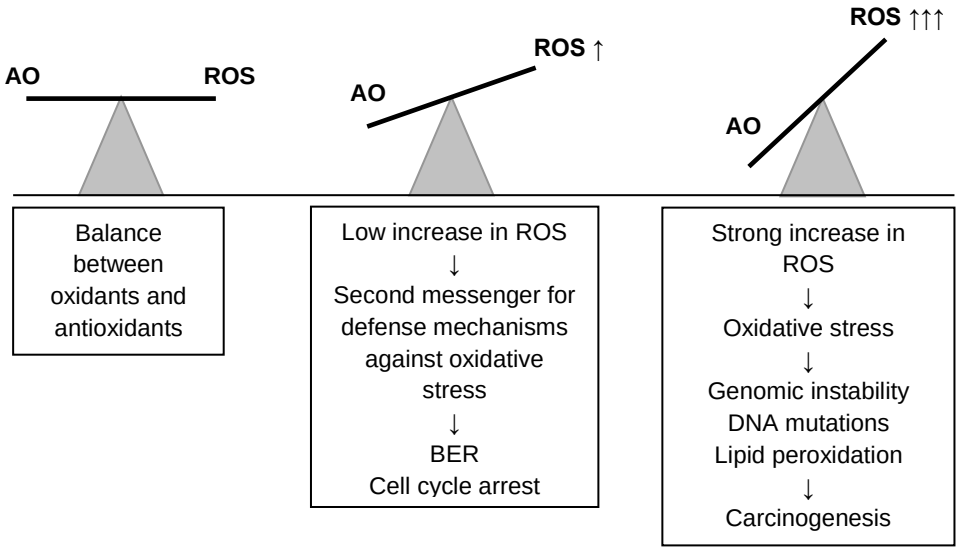

Figure 2 Dose response effects of reactive oxygen species (ROS). AO = antioxidants, BER = Base Excision Repair

Low levels of ROS are crucial in several physiologic processes of the cell including proliferation, apoptosis, cell cycle arrest, cellular senescence and immune reactions (Figure 2) [33, 36]. Endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) that act on $O_2^{\bullet-}$ and H_2O_2, respectively, and glutathione peroxidase (Gpx1) that uses glutathione as co-substrate play an important role in the protection of the cell to changes in redox balance. The primary and most crucial step in the defense against ROS includes the activation of nuclear factor (erythroid-derived 2)-like 2 (NRF2). This transcription factor binds to the antioxidant response element together with small MAF (MAFG and MAFK) to activate transcription of antioxidant scavengers such as superoxide dismutase (SOD1 and 2) and glutathione (GSH) (Figure 3) [37]. NRF2 is ubiquitously expressed and is retained and degraded in the cytoplasm under normal cellular conditions by active cysteine residues from the Kelch-like ECH-associated protein 1 (KEAP1). When cells are under oxidative stress, the formation of this NRF2/KEAP1 complex is inhibited and NRF2 is transferred to the nucleus (Figure 3). Oxidative stress can induce this NRF2 activation directly by modifying KEAP1 to decrease ubiquitination and degradation of NRF2 [38] or by oxidizing cysteine residues from KEAP1 [39] and therefore promotes its nuclear importation. Alternatively, oxidative stress is capable of inducing expression of different kinases such as ERK, JNK, PKC and PI3K/AKT that can phosphorylate NRF2, which leads to dissociation from KEAP1 and subsequent nuclear transportation in this way [40-42] (Figure 3).

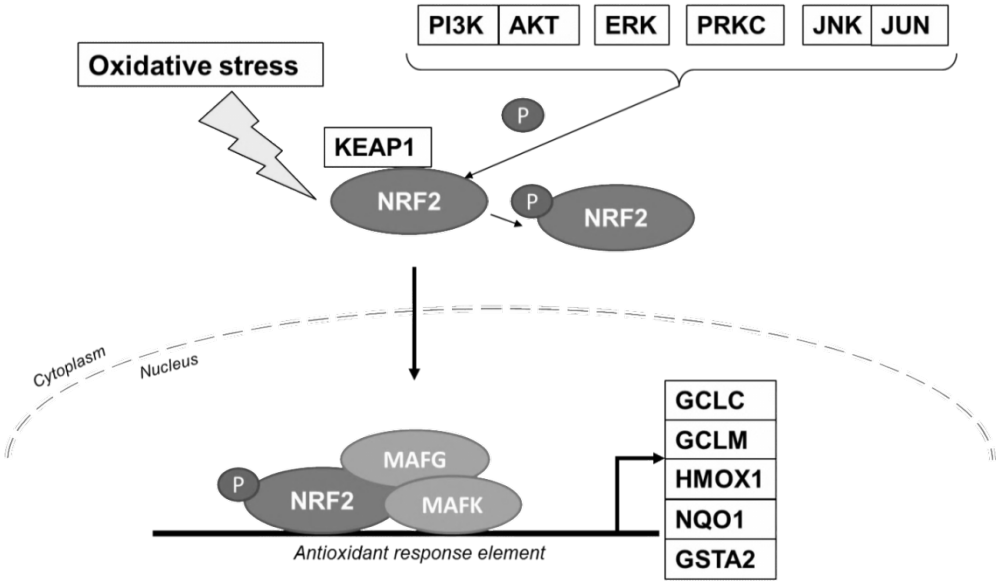

Figure 3 Schematic overview of NRF2 activation to induce phase II response genes (GCLC, GCLM, HMOX1, NQO1 and GSTA2) by escape from KEAP1 and transportation to the nucleus after direct activation by oxidative stress or by phosphorylation through PI3K, ERK, PRKC or JNK activity. NRF2 binds to the antioxidant responsive element together with small maf (MAFG and MAFK) to activate the antioxidant machinery.

In addition, nuclear factor-κB (NF-κB) is activated by an upstream signaling element, Ras, and is involved in the activation of other antioxidant genes [43]. If these defense mechanisms against increased ROS levels fail, oxidative damage in DNA, proteins, lipids and other cellular components may occur. This leads to the activation of other defense mechanisms such as DNA repair by base excision repair (BER). 8-oxo-2'-deoxyguanosine (8-OHdG), a fingerprint of oxidative DNA damage, is the most prevalent and deleterious DNA base lesion induced by oxidative stress because it preferentially mispairs with adenine during replication [44]. When first line DNA repair mechanisms such as human MutT homologue 1 (hMTH1) and human 8-oxo-guanine DNA glycosylase (hOGG1) are ineffective in avoiding mutation [45, 46], mispairing results in G:C to T:A transversion [44]. This mutation however, can be recognized by BER [47], and a p53-mediated cell cycle arrest occurs which inhibits DNA synthesis to allow the DNA repair machinery time for correctly repairing the damage [48, 49]. In case DNA damage is not repaired by these mechanisms, apoptosis is induced to avoid passage of DNA mutations to progeny cells. This cascade of events in response to intracellular ROS challenge is highly dynamic and may induce diverse sequential changes in cellular response.

Exogenous sources such as environmental agents, including liver carcinogens, on the other hand, can excessively elevate ROS levels and create a toxic environment for the cells by inducing high chronic oxidative stress [32] (Figure

2). This stressful condition plays a major role in cancer development and promotion by mainly enhancing DNA damage and modifying key cellular processes as will be described in following paragraphs.

Hepatocarcinogenesis and oxidative stress

Spontaneous mutations that are induced by oxidative stress may initiate carcinogenesis [50], and various cancers, including HCC, have been found to be in a constant state of oxidative stress, which suggests a role for oxidative stress in cancer promotion as well [51]. As a portal of entry for xenobiotics, the liver is continuously exposed to multiple chemicals, and as such is prone to oxidative-induced damage by different types of oxidative agents which will be further discussed in paragraph 2.3. Consequently, the imbalance between ROS formation and antioxidant capacity in the liver has been linked to increased cancer risk [52, 53]. In the liver, mitochondrial and cytochrome P450 enzymes of hepatocytes, endotoxin-activated Kupffer cells, and neutrophils represent the main sources of free oxygen radicals. ROS are involved in transcription and activation of different cytokines and growth factors, and play therefore a crucial role in the pathogenesis and the progression of HCC [54]; for example, the increased presence of 8-OHdG lesions correlates with a higher risk for HCC development which indicates that oxidative DNA damage induced by ROS leads to hepatocarcinogenesis [55, 56]. ROS-induced mechanisms have actually been related to different chronic liver diseases and HCC, and are induced by various risk factors for liver cancer such as hepatitis B and C or aflatoxin-B1 (AFB1) [17]. Moreover, similar mechanisms are involved in the activation of intrinsic drug resistance genes in HCC.

Elevated 8-OHdG levels and lipid peroxidation biomarkers were detected in the liver of animals carrying Hepatitis B or C infections, which provides additional evidence for the association between oxidative stress and hepatocarcinogenesis [57]. Furthermore, different hepatocarcinogens, AFB1 (see also paragraph 2.3) and 2-acetylaminofluorene (2-AAF) have been found to induce oxidative DNA damage in cultured hepatocytes [58, 59]. However, the most relevant model supporting the role of ROS in hepatocarcinogenesis, is presented by the transgenic sod1(-/-) mouse, which exhibits oxidative DNA damage accompanied with an increased incidence of liver malignancies [60].

These oxidative DNA adducts formed by ROS, may promote mutations during DNA replication. Furthermore, ROS-induced single-strand breaks, can accumulate in the cell over time and evolve into double-strand breaks [61] making chromosomes vulnerable to translocations leading to genomic instability. Persistent intracellular ROS induce accumulating DNA-, RNA-, protein- and lipid damage resulting in altering gene expression patterns and different phenotypes, ultimately leading to malignant transformations in the liver. High activity of oxidative stress-responsive transcription factors NRF2 and NF-κB was observed in patients with HCC, as well as other redox regulating transcription factors including FOS, JUN, STAT, AP1 and AP2 [62-64]. In addition, different cellular signaling cascades,

activated by oxidative stress, have been found to be relevant in HCC such as Wnt/β-catenin as previously described (paragraph 1.3.2) [65]. However, in general, knowledge of these sequential oxidative stress-related responses leading to liver toxicity and HCC is still fragmentary and hampers a reliable prediction of human health risks towards new chemicals and drugs.

Liver carcinogens and oxidative stress

As previously described, HCC can be induced by different types of chemicals present in the environment. AFB1 is the best described liver carcinogen in humans but many others exist. These chemical carcinogens are classified as either genotoxic (GTX) or nongenotoxic (NGTX) based on mechanistic information [66]. Mechanisms of carcinogenic capacities are not fully understood for most chemicals; especially knowledge of the involved mechanisms for NGTX carcinogens is considerably less extensive than for GTX carcinogens. NGTX carcinogens are chemicals that induce malignancies without reacting directly with DNA, and probably act as tumor promoters. This is in contrast to GTX carcinogens, which are DNA reactive, act as tumor initiators, and are assumed to exhibit proportional responses at low doses. Thus, GTX agents produce chemical alterations in DNA directly, whereas NGTX agents are thought to indirectly stimulate hyperplastic or neoplastic responses [67-69]. A number of effects induced by these hepatocarcinogens contribute to the manifestation of HCC and one important factor in appears to be the formation of intracellular ROS and subsequent induction of oxidative stress [69, 70]. Results from rat studies suggest that oxidative stress plays a key role in the mode of action of NGTX carcinogens [71-73]. Enzyme systems such as the NADPH-oxidase complex, cyclooxygenase, xanthine-oxidase, lipoxygenase, cytochrome-P450 and peroxisomes, as well as inflammatory cells are sources of endogenous ROS [32, 70] which may be affected by NGTX carcinogens.

NGTX carcinogens such as different types of dioxins, may cause different cytochrome p450 enzymes to leak ROS and thus promote cell division, lipid peroxidation, DNA single-strand breaks, modified base formation, decrease in membrane fluidity, and decreases in hepatic GSH and NADPH content [68]. Dioxins and other NGTX compounds bind with high affinity to the aryl hydrocarbon receptor (AhR) [68, 74]. AhR is an important homeostatic transcriptional regulator within physiological and pathophysiological processes, including xenobiotic metabolism and cancer [74]. The ligand bound receptor translocates to the nucleus where it binds to its heterodimeric partner, the aryl hydrocarbon nuclear translocator (ARNT) [74]. The AhR/ARNT complex functions as a transcription factor upon binding enhancer sequences, xenobiotic response elements (XREs), found upstream of a number of genes, including cytochrome p450 family genes [68]. Cytochrome p450 activation may then cause oxidative stress.

NGTX carcinogens may also induce intracellular ROS formation by other modes of action. For example, Tetradecanoyl-phorbol-acetate (TPA), a NGTX tumor promoter, exerts its toxic effects by activation of protein kinase C (PKC) and

integrins which may lead to ROS formation by NADPH oxidase activity [75]. Another example is tetrachloroethylene (TCE), which is able to deplete glutathione (GSH), and thus causes that intracellular ROS is not captured anymore [76].

The fact that NGTX mechanisms such as cytochrome p450, AhR binding, PKC activation and GSH depletion, imply ROS formation, highlights that oxidative stress is an import key player in hepatocarcinogenic events induced by these NGTX compounds. However, it is not clear whether these oxidative stress-related mechanisms are limited to NGTX carcinogens or differ between particular carcinogens, *e.g.*, GTX and NGTX carcinogenesis.

Epigenetics and oxidative stress

Recently, it has been shown that cellular responses towards oxidative stress are not only limited to direct gene expression changes, but also epigenetic processes can be disturbed [77-81]. In biology, and specifically in genetics, epigenetics is the study of relevant changes in the genome that are not caused by changes in the DNA sequence. Examples of mechanisms that produce such changes are DNA methylation and histone modification, each of which can change gene expression without altering the underlying DNA sequence. These epigenetic markings on DNA, such as DNA methylation, are crucial for establishing tissue-specific gene expression that distinguishes different types of cells and for the maintenance of normal cellular function. Alternatively, disruption of DNA methylation and demethylation, by factors such as oxidative stress can contribute to genomic instability and therefore to the promotion of cancers, including HCC [77-81]. However, how oxidative stress can induce aberrant DNA methylation and demethylation is not clear.

DNA methylation, especially in the promoter or exon 1 region, plays an important role in transcriptional regulation of genes by enzymatically addition or deletion of methyl groups [82-84]. The well-known methylated cytosine base, 5-methylcytosine (5mC), is described as the fifth base of the genome and is highly important in embryogenic development. Methylation of DNA and histones occurs through the addition of methyl groups, by DNA methyltransferase (DNMT) enzymes or histone methyltransferases respectively, using S-adenosylmethionine (SAM) as methyl donor [81]. On the other hand, these methyl groups can also be deleted from DNA by passive and active mechanisms. These demethylation processes however, are less well understood. One of these active demethylation mechanisms has been recently discovered in the form of oxidized 5mC, 5-hydroxymethylcytosine (5hmC), which is now considered to be the sixth base in DNA [85]. Particular enzymes, ten-eleven translocation 1, 2 and 3 (TET1-3), have been found to oxidize 5mC to form 5hmC. Moreover, these different TET enzymes are capable of further oxidizing 5hmC into 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC). These oxidation products are suggested to be intermediates in the removal of 5mC [86, 87]. Subsequently, these oxidation products are either actively removed through BER or passively through replication-

dependent depletion of these modified bases [88, 89]. Therefore, 5hmC, together with these TET enzymes, plays a significant role in epigenetic reprogramming and cellular differentiation by regulating tissue specific gene expression.

The accumulation of both 5mC and 5hmC modifications has been observed in HCC, and is considered to play an important role in the alteration of cancer-related genes in the development of HCC [21]. In more detail, epigenetic studies demonstrated that global hypomethylation and promoter hypermethylation may be early events in the development of HCC. These studies propose that oxidative stress can induce transcriptional silencing of tumor suppressor genes, such as Socs-1 and E-cadherin, in HCC by hypermethylation of their promoter regions [79, 80]. It is suggested that these oxidative stress-induced alterations in DNA methylation status are regulated by affecting the activity of DNMTs [77, 78]. On the other hand, how oxidative stress can contribute to changes in 5hmC status is less understood. Recently, it has been shown that $HO^{\bullet}$ can oxidize 5mC directly without the action of TET enzymes [90]. How this may contribute to gene expression changes and active demethylation, however, is not clear. Lastly, it is not known whether established liver carcinogens induce epigenetic changes which may be crucial in hepatocarcinogenesis, and if so, oxidative stress contributes to this.

Toxicological experiments using different 'omics approaches

To obtain better insights in these previously described oxidative stress-related mechanisms in the formation of hepatotoxicity and –carcinogenesis, it is important to explore whole genome changes induced by oxidative compounds. The completion of the human genome project in 2003 triggered the rapid development of so-called 'omics technologies in the field of molecular biology [91]. These 'omics technologies comprise multiple platforms ranging from genomics (focusing on the genome) to proteomics (focusing on the proteome) and metabolomics (focusing on the metabolome). These technologies can be applied in different research fields, for instance, the study of changed whole genome gene expression patterns induced by toxicants is called toxicogenomics. Since many parameters can be measured simultaneously using these techniques, additional information in the molecular mechanisms behind oxidative stress can be provided.

Information about transcription of almost all genes can be obtained by genomics, which is named transcriptomics. Transcriptomics provides us with a quantitative overview of the mRNA transcripts that were present in a sample at the time of preparation. In parallel, the epigenetic status, such as DNA (hydroxy)methylation, of the same genes can be evaluated, using epigenomic technologies. Epigenomics, or more specific (hydroxy)methylomics, provides us with information about differentially (hydroxy)methylated regions at the DNA level.

In transcriptomics, mRNA is isolated from compound-treated or untreated cells or tissue. This material is amplified into fluorescent labeled cRNA or cDNA and is hybridized to a large set of oligonucleotide probes which are spotted onto glass slides (=microarray). These probes consist out of RNA sequences that accounts for a specific gene. When labeling control and exposed cRNA or cDNA with different colors and bind to probes on the same array, the signals that are detected in a scanner, can then be compared to each other and differences in transcription of specific genes can be calculated (Figure 4). Alternatively, one-color approaches do exist were each sample (control or exposed) is labeled in an identical way, but hybridized to separate arrays [92]. Different types of arrays exist and use these different hybridization techniques, however, a high level of concordance between these different platforms exist, making it possible to perform inter-platform comparative studies [93].

In a similar way as in transcriptomics, (hydroxy)methylomics measurements are performed. For this, DNA is isolated and immunoprecipitated using 5mC or 5hmC specific antibodies to capture specific methylated or hydroxymethylated DNA sequences respectively (Figure 5). These methylated or hydroxymethylated DNA fractions are then fluorescently labeled and spotted onto specifically designed array platforms carrying small probes (~50 base pairs) which represent sequences in the genome that are within biologically focused regions such as promoter or CpG islands. In this way, the genomic distribution and changes in 5mC and 5hmC can be analyzed after exposure to different types of compounds.

Data resulting from both these types of measurements can be analyzed using a wide range of bioinformatics tools, such as specific pathway analysis tools or correlation analysis.

Overall, these analyses can be used to determine which genes are differentially expressed or methylated as a result of compound treatment. Combining these toxicogenomics approaches, may therefore provide a good model for increasing our knowledge about dynamic oxidative stress-related mechanisms.

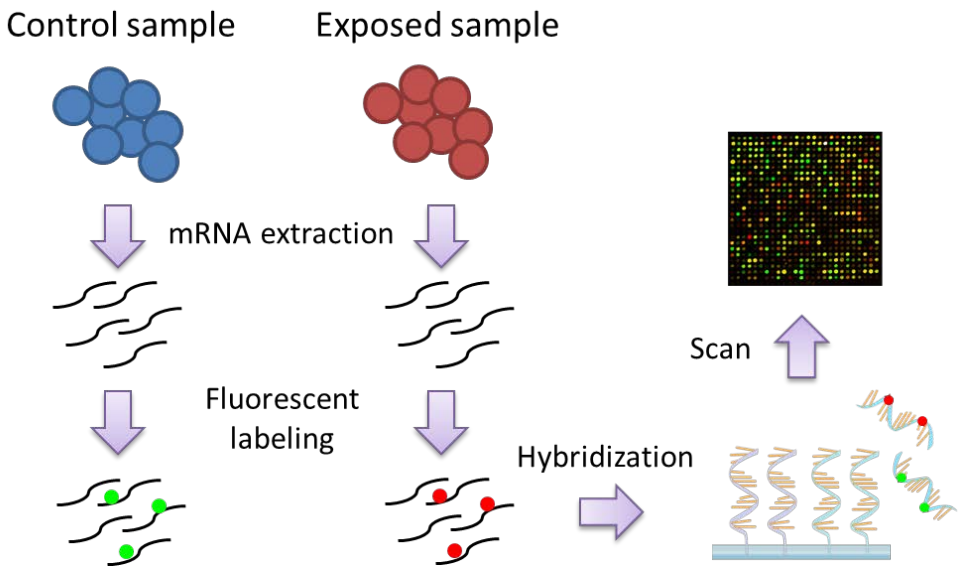

Figure 4 DNA microarray hybridization using two colors. Figure adapted from Grigoryev [94]

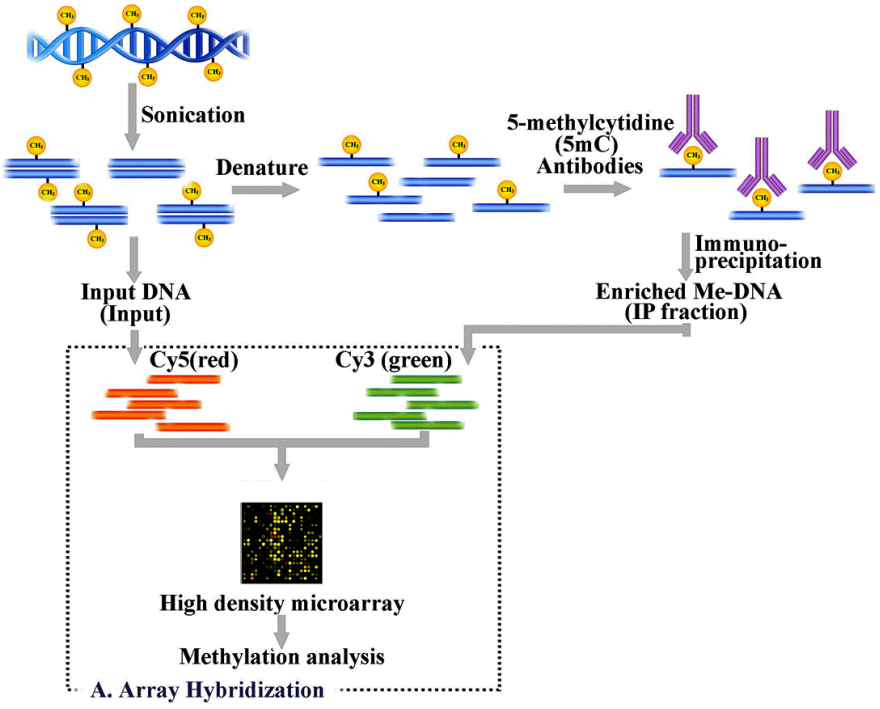

Figure 5 Schematic outline of mehylcytosine immunoprecipitation. DNA is sonicated and denatured. Incubation with 5mC (or 5hmC) antibodies, along with immunoprecipitation, to obtain methylated fragments. Fractions are labeled and hybridized to high-density microarrays. Adapted from http://commons.wikimedia.org/wiki/File:Medip-final-3.jpg.

Time series analysis in toxicogenomics

As already mentioned, biological responses towards oxidative stress are variable across time. Therefore, applying extensive time-series experiments are important to investigate the oxidative stress-induced responses of both the transcriptome and (hydroxy)methylome as well as their interplay over time [95, 96]. In these types of experiments, different data points are measured at early but also at later time points following exposure to different types of oxidative compounds. In this way, we are capable of discriminating between early acute responses and later, more persistent, cellular responses [97]. Temporal gene expression data can thus be used to gain a wide range of insights, such as characterizing the function of specific genes across time and their relation with other genes. Also, by comparing time courses of co-regulated genes with time courses of functional endpoints (i.e. biomarkers for cellular damage induced by oxidative stress), additional insight in oxidative stress-related responses can be obtained (= Phenotypic anchoring) [98, 99] since different genes and functional endpoints that correlate across time following exposure are assumed to be functionally related [95]. Another benefit of using time series gene expression data, instead of static measurements, is the ability to capture information about transient gene expression changes which would be missed if only one measurement over time is considered. Upon inducing exposure, different sets of genes may respond with different kinetics, and therefore, by using this approach, a complete set of events can be captured by measuring gene expression at multiple time points [95].

The use of *in vitro* systems easily enables these types of temporal experiments. These *in vitro* tests are quick and relatively inexpensive, and provide us with the ability to measure specific functional endpoints. Currently used *in vitro* liver systems are cell lines such as HepG2 cells, HepaRG cells, precision-cut liver slices and primary hepatocytes. Although primary hepatocytes and liver slices resemble the *in vivo* situation best, they exhibit high individual variability in gene expression, are expensive and less easy to handle than immortalized cell lines. HepG2 cells have been used frequently in toxicological studies. Together, the ability of HepG2 cells to carry out biotransformation of xenobiotics [100], although with a lower metabolic capacity compared to hepatocytes [101], the absence of p53 mutations [102], and their easy usability in comparison to, for example, primary human hepatocytes, make these cells a convenient alternative for *in vivo* testing [103].

Aim and outline of the thesis

Human disease risks and high economic costs due to increasing HCC incidence can partly be avoided by early detection. Better insights in previously described molecular mechanisms of risk factors, such as oxidative stress, in the formation of hepatocarcinogenesis are therefore of major importance. Based on different studies during the last decades, it is known that oxidative stress status has

a key role in HCC development and progression. Different studies have shown that oxidative stress can be induced directly by exposure to oxidative compounds, as well as by endogenously mechanisms in liver cells. However, insights in molecular mechanisms which explain how ROS induce malignant formation and uncontrollable growth in HCC remain sparse.

Application of 'omics technologies may provide in depth knowledge of underlying molecular events. However, so far, no studies in liver cells have been published which apply an extensive time series analysis including early time points, in order to understand common and unique sequential cellular and (epi-)genetic changes upon exposure to a range different types of chemical carcinogens. By applying such temporal 'omics analyses *in vitro*, this thesis aims to improve the understanding of the molecular mechanisms of oxidative stress in relation to hepatocarcinogenesis.

The use of microarray-based whole genome gene expression and DNA methylation analysis in combination with functional endpoints such as (oxidative-) DNA damage, cell cycle distribution, apoptosis may provide answers to important issues concerning oxidative stress-induced hepatocarcinogenesis. In addition, by using a methylomics approach, which provides us with the ability to integrate whole genome DNA methylation levels with whole genome gene expression, new insights into the sequential cross-talk between genetic and epigenetic response towards oxidative stress can be obtained. Overall, oxidative stress-specific signatures can be identified which may improve hazard identification of new and existing chemicals and pharmaceuticals.

This thesis is thus based on the hypothesis that oxidative stress-induced (epi)genetic modifications play an in important role in human HCC development and promotion. Therefore, the aim of this thesis is to obtain whole genome gene expression and methylation changes in combination with markers of oxidative stress-induced damage in a human hepatoma cell line (HepG2), to gain better insights in the carcinogenic impact of oxidant exposure, with particular emphasis on HCC.

To realize this objective, HepG2 cells were challenged by different types of oxidative agents, menadione, *tert*-butyl hydroperoxide and hydrogen peroxide, across time, in order to understand sequential changes in gene expression responses to different types of oxygen radicals; this is described in **Chapter 2**. Phenotypic anchoring between gene expression data and functional endpoints was performed to offer a better understanding of differentially expressed molecular pathways leading to cellular toxicity or cancer-related events.

In **Chapter 3** results from HepG2 cells obtained in Chapter 2 were compared with gene expression data from Caco-2 cells, a human colon carcinoma cell line, exposed to both superoxide and hydroxyl radicals. Knowledge about possible organ-specific oxidative stress-mechanisms is limited. Therefore, we evaluated whether these oxidative stress-related mechanisms as observed in Chapter 2, were liver-specific.

In **Chapter 4,** we used the oxidative stress-related signature of seventeen genes obtained in Chapter 2, to select compounds with regard to their ability to induce oxidative stress. As most of these genes are involved in hepatocarcinogenes and/or oxidative stress, it was of interest to investigate the relation between upregulation of these genes and oxidative stress induced by NGTX carcinogens in comparison to GTX carcinogens. Therefore, in this study, oxidative stress-related mechanisms were compared between selected GTX-, NGTX- and non-carcinogens to evaluate the role of oxidative stress in NGTX carcinogens in the formation of HCC.

In the next two chapters, we focused on DNA methylation in combination with gene expression changes induced by *tert*-butyl hydroperoxide (TBH). Both accumulations of genetic and epigenetic alterations are observed in HCC, and are considered to play a crucial role in changing expression of cancer-related genes for the development of HCC. In **Chapter 5,** the interplay between transcriptomics and methylomics responses to TBH exposure in HepG2 cells were analyzed to obtain a better understanding about sequential cross-talk between gene expression and 5mC status following oxidative stress. This approach gives us the opportunity to identify DNA methylation marks in promoter of exon 1 of strongly induced genes to investigate interplay between transcriptomic and methylomic responses towards TBH challenge.

In **Chapter 6,** the formation and distribution of 5hmC by TBH treatment was investigated. Different oxidation products of 5mC were chemically identified. This showed an increase in 5hmC levels induced by oxidative stress. The distribution and effects of these 5hmC adducts were further analyzed by integrating this data with transcriptomics and methylomics to evaluate whether these 5hmC modifications were directly formed by oxidative stress or by enzymatic interference of TET proteins.

Chapter 7 presents a summary and general discussion of the major findings of all studies presented in this thesis and future perspectives are discussed.

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CHAPTER 2

Time series analysis of oxidative stress response patterns in HepG2: A toxicogenomics approach

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Abstract

Oxidative stress plays an important role in chemically induced liver injury, however, our insight into molecular responses to different oxygen radicals is fragmentary. Since these cellular responses will differ over time, examining time-dependent changes in gene expression, and correlating these with markers for oxidative stress, may provide new insights into responses to oxidants. We used the human hepatoma cell line HepG2 to investigate the effects of oxidative stress on the transcriptome level by micro-arrays at seven time points (0.5, 1, 2, 4, 6, 8, 24h) following exposure to the oxidants menadione, hydrogen peroxide and *tert*-butyl hydroperoxide including the effects on cell cycle and apoptosis by flow cytometry, protein carbonyl formation by spectrophotometry and oxidative DNA damage by FPG-comet. In total, 3429 genes were differentially expressed, including 136 genes that were significantly modified by all oxidants. Time-dependent biological pathway analysis showed that these genes were particularly involved in inflammatory responses, cell cycle processes and glutathione signaling. These responses where confirmed and supported by phenotypic anchoring to the different cellular endpoints. In addition, using an innovative temporal analysis we established an oxidative stress-related gene expression time cluster.

Altogether, this study provides new insights in temporal oxidative stress mechanisms and demonstrates sequential cellular responses that may contribute to a better hazard identification and the mechanisms of toxicological responses in the liver induced by oxidative stress.

Introduction

A proper balance between the formation of reactive oxygen species (ROS) and the antioxidant network is known to be essential for regulation of biological processes [1, 2]. However, uncontrolled ROS formation results in oxidative stress in cells, which subsequently leads to damage to different cell structures such as DNA, proteins and lipids [3]. ROS-induced mechanisms have for instance been related to different chronic liver diseases and hepatocellular carcinoma (HCC), and are induced by various risk factors for liver cancer such as hepatitis B and C or aflatoxin-B1 [4], and thus may be a possible driving force in hepatocarcinogenesis [5].

Various relevant forms of ROS exist, namely superoxide anion radicals ($O_2^{\bullet}$), hydroxyl radicals ($^{\bullet}OH$), hydrogen peroxide and peroxides, they affect cells differently, react differently with diverse antioxidant enzymes, and have different preferred cellular targets. Furthermore, low ROS levels act as second messengers to promote gene expression of defense mechanisms for oxidative stress induced injury to cells [2]. The primary and most crucial step in the defense against ROS includes the expression of nuclear factor (erythroid-derived 2)-like 2 (NRF2) which promotes expression of antioxidant scavengers, *e.g.*, superoxide dismutase (SOD1, 2 and 3) and glutathione (GSH) by binding to the antioxidant response elements (ARE) [6]. In addition, nuclear factor-κB (NF-κB) is activated by an upstream signaling element, Ras, and is involved in activation of other antioxidant genes [7]. If these defense mechanisms against increased ROS levels fail, oxidative DNA damage may occur and may lead to mutations. DNA damage can be detected and repaired by several mechanisms, or, when not repaired, apoptosis is induced to avoid the passing on of DNA mutations to progeny cells. However, exogenous sources such as environmental agents can excessively elevate ROS levels and induce high levels of chronic oxidative stress in hepatocytes, which may, in turn, cause fixed DNA mutations [8].

The still fragmentary knowledge of these cellular stress responses to different types of oxygen radicals leading to different cellular damage mechanisms hampers a reliable prediction of human health risks for chronic liver toxicity and carcinogenesis of new chemicals and drugs. Since such cellular responses will differ in time, examining time-dependent, sequential changes in gene expression may provide new insights in responses to oxidants, the underlying hypothesis being that gene expression modifications which cluster in time are functionally inter-related. Additionally, phenotypical anchoring between gene expression data and functional endpoints will offer a better understanding of differentially expressed molecular pathways leading to cellular toxicity or cancer-related events. A human hepatoma cell line (HepG2 cells) was used, because hepatocytes are the major site of xenobiotic metabolism within the liver and hence represent the most important cellular target for toxic reactive metabolites. Together, the ability of HepG2 cells to carry out biotransformation of xenobiotics [9], although with a lower

metabolic capacity compared to hepatocytes [10], the absence of p53 mutations [11], and their easy usability in comparison to, for example, primary human hepatocytes, make these cells a convenient alternative for *in vivo* testing of *e.g.*, genotoxic compounds [12]. Global gene expression profiling in HepG2 cells was performed by micro-array-based experiments by applying extensive time series analyses following exposure to menadione, tert-butyl hydroperoxide (TBH) and hydrogen peroxide (H_2O_2). Oxidant-induced radical formation by the selected compounds was identified and quantified by using electron spin resonance (ESR) spectroscopy. In addition, to correlate whole-genome gene expression to markers for oxidative stress, (oxidative) DNA damage, protein oxidation, as well as apoptosis and cell cycle progression were measured.

Material and methods

Cell culture

HepG2 cells (ATCC, LGC logistics) were cultured in 6-well plates in the presence of minimal essential medium supplemented with 1% nonessential amino acids, 1% sodium pyruvate, 2% penicillin/streptomycin, and 10% fetal bovine serum (all from Gibco BRL, Breda, The Netherlands). The cells were incubated at 37°C and 5% CO2. When cells were 80% confluent, the medium was replaced with medium containing Menadione (Sigma-Aldrich, Zwijndrecht, The Netherlands), TBH (Sigma-Aldrich) or H_2O_2 (VWR int, UK) in combination with Fe^{2+} ions (derived from $FeSO_4$) (Merck-Millipore, Germany). As a solvent control, medium was used. Time-matched control cells were treated in an identical manner without addition of any oxidants.

Identification and levels of radical formation

Radical formation in HepG2 cells was measured by ESR spectroscopy in combination with the spin trapping technique using 50 mM 5,5-dimethyl-1-pyrolline N-oxide (DMPO, Sigma-Aldrich) as described before [13].

Dose selection

Non-cytotoxic concentrations were selected based on a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cytotoxicity assay (80% viability) after 24h exposure in 96-well plates for the highest dose [14]. In addition, using ESR spectroscopy, final concentrations (100 µM menadione, 200 µM TBH and 50 µM H_2O_2/Fe^2) were determined based on maximum oxygen radical formation at a non-cytotoxic dose.

Total RNA Isolation and real time PCR

Total RNA was extracted using 0.5 ml QIAZOL according to the manufacturer's instructions. MiRNeasy Mini Kits (Qiagen, Westburg, The Netherlands) were used to purify total RNA. RNA quantity and purity were

determined spectrophotometrically. RNA quality was assessed by automated gel electrophoresis on an Agilent 2100 Bioanalyzer (Agilent Technologies, Amstelveen, The Netherlands). Extracted RNA was stored at −80°C until it was used as template for complementary DNA (cDNA) synthesis.

Quantitative real-time (RT) PCR was performed in duplicate as previously reported [15]. Beta-Actin messenger RNA was used as a reference. RT-PCR was run on the MyiQ Single-Color RT-PCR Detection System (Bio-Rad Laboratories). SOD1, SOD2, catalase and beta-actin, forward and reverse primers were previously described [16]. Other primers used: CDKN1A 5'-GCAGACCAGCATGACAGATTTC-3'/3'-GCGGATTAGGGCTTCCTCTT-5', OGG1 5'-AGAGGTGGCTCAGAAATTCCAA-3'/3'-CAGATAAAAGAGAAAAGGCATTCGA-5', GPX1 5'-CCCGTGCAACCAGTTTGG-3'/3'-CGGACGTACTTGAGGGAATTCA-5', HMOX1 5'-CTTCTTCACCTTCCCCAACA-3'/3'-GCTCTGGTCCTTGGTGTCAT. Gene expression was calculated using the $\Delta\Delta$Ct log method and log base 2 transformed [17] for correlation analysis with gene expression.

Apoptosis and cell cycle distribution

Analyses of cell cycle profiles and apoptosis were performed using flow cytometry [15]. Cells were stained with propidium-iodide; apoptotic cells were visualized by the primary antibody M30 CytoDeath (Roche, Penzberg, Germany) and fluorescein isothiocyanate conjugated anti-mouse Ig as secondary antibody (DakoCytomation, Glostrup, Denmark). Data analysis was performed as previously described [16].

Protein carbonyl assay

Following exposure to menadione, TBH or H_2O_2/Fe^{2+} at indicated time points in T75 flasks, proteins were extracted in RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50mM Tris, pH 7.5). Protein concentrations were determined by the Lowry assay (Bio-Rad) with bovine serum albumin as a standard. Levels of oxidized proteins in cells were measured using the protein carbonyl assay kit with 2,4-Dinitrophenylhydrazine (DNPH) as previously described [18] (Protein carbonyl colorimetric assay kit, Cayman Chemicals, USA). The carbonyl content was calculated from the spectrophotometric absorbance measured at 370 nm using an absorption coefficient of 22,000M^{-1} cm^{-1}.

Oxidative DNA damage measurements

Cells were harvested by trypsinization and resuspended in growth medium. Cells were centrifuged (300g, 5min), washed once in PBS, and placed on ice. A standard and formamido pyrimidine glycosylase (Fpg) protein alkaline comet assays (Fpg, New England Biolabs, England) were subsequently performed in triplicate as described by Singh [19] and Pflaum [20] with minor modifications. Comets were visualized using a Zeiss Axioskop fluorescence microscope (at 200x

magnification). Randomly, 50 cells were analyzed using the Comet assay III software (Perceptive Instruments, Haverhill, UK). DNA damage was expressed as tail moment (TM, the product of tail DNA content and mean tail migration distance).

Whole genome gene expression

cDNA was prepared using Affymetrix synthesis and labeling kits as described before (Affymetrix, Santa Clara) [9]. cRNA targets were hybridized on high-density oligonucleotide genetitan chips (Affymetrix Human Genome U133 Plus PM GeneTitan 24 arrays) according to the Affymetrix Eukaryotic Target Hybridization manual. The GeneTitan arrays were hybridized, washed and stained using the GeneTitan hybridization, wash and stain kit for 3' IVT Arrays and GeneTitan Operating Software and scanned by means of an Affymetrix GeneTitan scanner. Normalization quality controls, including scaling factors, average intensities, present calls, background intensities, noise, and raw Q values, were within acceptable limits for all chips.

Whole genome gene expression data analysis:

Re-annotation and normalization

Data from one hundred and twenty six arrays were obtained, and Robust Multi-array Average (RMA) normalized and re-annotated to custom CDF files using the arrayanalysis tool (http://arrayanalysis.org/). In addition, 18,909 genes were analyzed for the number of absence calls in the three replicates per treatment. Genes that contained two or more absence calls within the three replicates for all the treatments as well as in controls were omitted from the data.

The data discussed in this publication have been deposited in NCBI's gene expression omnibus [21] and are accessible through GEO series accession number GSE39291
http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE39291

Data filtering and clustering

The intensities of the filtered data sets were log2 transformed, and subsequently, log ratios of treated versus controls were calculated. Differentially expressed genes (DEGs) for each experimental group were selected using the following criteria: (1) log ratio of -0.26 or >0.26 (i.e., absolute fold change of 1.2) for the average of the three replicates within the experimental group, (2) same direction of the log ratio for all replicates, (3) intensity of log2 values >6 for at least 2 out of 3 replicates, and (4) a p value of <0.05 determined using the student's t-test. No FDR was used since it has been reported that reproducibility of microarray data is higher when criteria such as fold change are used [22]. Overlapping DEGs for all compounds were clustered using the Hierarchical Clustering Analysis (HCA; Pearson correlation, Euclidean distance, pair wise complete distance) in GenePattern v.3.2.1 (http://genepattern.broadinstitute.org).

Time series analyses by STEM

For identification of genes co-regulated time-dependently and clustering with the markers for oxidative stress, the software tool ''Short Time-series Expression Miner'' (STEM, version 1.1.2b; http://www.cs.cmu.edu/~jernst/stem/) [23] was used. Criteria used were described before [16]. For correlation analysis, data from protein oxidation, 8-oxodG and cell cycle distribution levels were transformed into log 2 base ratios.

Pathway analysis of DEGs

MetaCore (GeneGo, San Diego, CA) was used to identify and visualize the involvement of the differentially expressed genes and transcription factors in the biological processes that may be affected at the level of pathways, by selecting significant pathways with a p value < 0.05.

Statistical Analyses of protein oxidation, oxidative DNA damage, apoptosis and Cell Cycle Effects

Data are presented as means ± SD. Statistical analyses of changes, for each time point compared to control, in apoptosis, protein oxidation, oxidative DNA damage, or cell cycle phases were performed using a paired two-tailed student's t-test with statistical significance set at $p < 0.05$.

Results

Dose and time point selection

HepG2 cells were exposed to a concentration range of menadione, TBH and H_2O_2/Fe^{2+}. Concentrations were chosen that showed a viability of >80% after 24 hours exposure in the MTT test (see supplementary data appendix A) combined with maximal oxygen radical formation as determined by ESR experiments. In addition, because no oxygen radical formation was observed after exposure to H_2O_2 alone, also $FeSO_4$ was added to the cells to provide Fe^{2+}. Based on these conditions, HepG2 cells were ultimately challenged by 100 µM menadione, 200 µM TBH or 50 µM H_2O_2/Fe^{2+}. Based on significant ($p<0.05$) gene expression changes established by RT-PCR data for SOD1 and 2, CAT, HMOX1, CDKN1A, GPX1 and OGG1, seven exposure time points were selected: 0.5, 1, 2, 4, 6, 8 and 24h (see supplementary data appendix B).

Radical formation

In the presence of HepG2 cells, menadione produced oxygen radicals ($DMPO^{\bullet}$-OH), most likely derived from superoxide anion [24], which increased over time (Figure 1) and were maintained until 8h after administration, while at 24h no ROS formation was detectable anymore. Exposure to TBH and H_2O_2/Fe^{2+} also led to significant cellular ROS formation ($DMPO^{\bullet}$-OH) probably derived from hydroxyl radicals. In addition, a methyl radical pattern was observed ($DMPO^{\bullet}$-CH3) after

exposure to TBH. At 0.5h significant DMPO•-OH signals were observed for all the oxidants. These signals slightly increased 2h after TBH administration but had disappeared 2h after starting exposure to H_2O_2/Fe^{2+}. In addition, at 0.5h after starting TBH treatment methyl radicals were generated, which were absent at 2h exposure. For all oxidants 24h after administration cellular ROS formation was not detectable anymore.

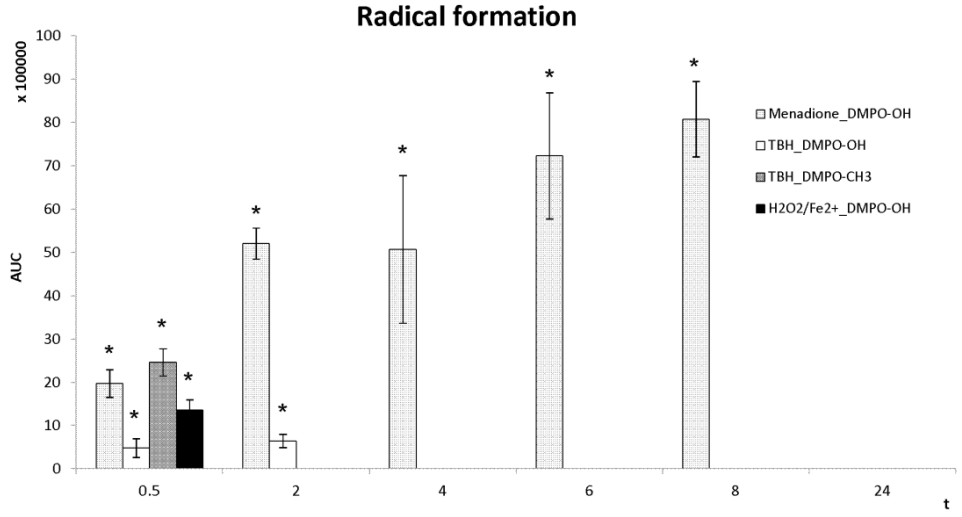

Figure 1 Levels of time dependent radical formation after exposure to menadione, TBH and H_2O_2/Fe^{2+} using ESR in combination with spin trapping by DMPO. Results are corrected for background levels observed in control conditions (n=3, $p<0.05$). AUC: area under the curve (x100000) of radical specific signals; t: time (h)

Apoptosis and changes in cell cycle distribution

Increased apoptosis was only found 24h after menadione administration while no apoptosis was detected after exposure to TBH or H_2O_2/Fe^{2+} (data not shown). Exposure to menadione had no effect on cell cycle distribution. However, TBH affected the cell cycle significantly at 6, 8 and 24h (Figure 2A-C) after administration by inducing a decrease in G1 phase and an increase in S phase compared to controls at these time points. Over time, a delay in cell cycle progression was observed. HepG2 cells exposed to H_2O_2/Fe^{2+} showed a significant increase in G2 levels after 6h administration compared to control at this time point.

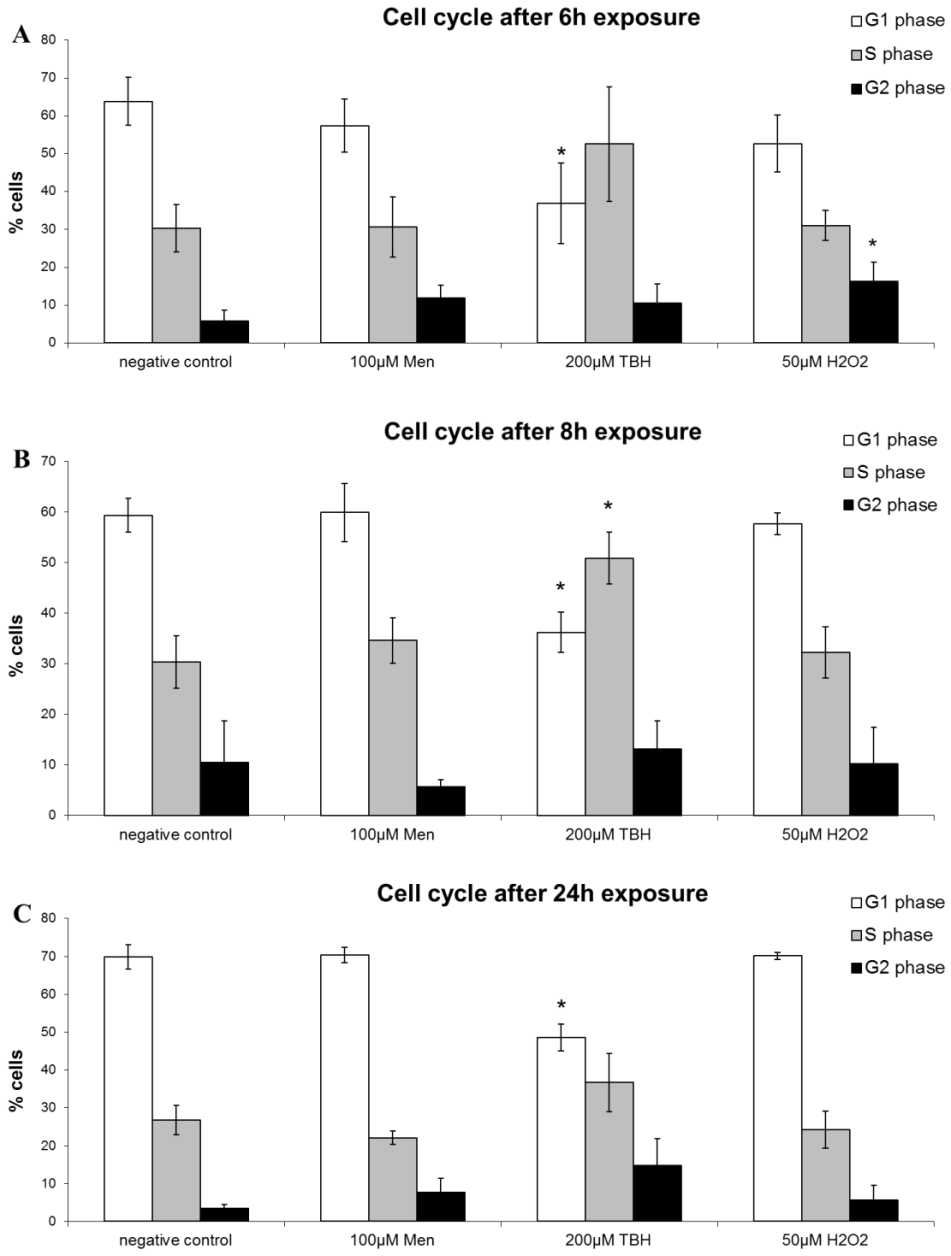

Figure 2 Levels of different cell cycle phases: G1 phase, S phase and G2/M phase. (A) Cell cycle distribution of HepG2 cells after exposure to menadione, TBH and H_2O_2/Fe^{2+} after 6h, (B) 8h exposure and (C) 24h exposure. (n=3, $p<0.05$)

Oxidant-induced protein carbonyl formation indicates protein oxidation

A significant increase in protein oxidation levels (carbonyl groups) compared to control samples was observed 6h after commencing exposure to

menadione (Figure 3A). After 8h administration to menadione, protein oxidation levels dropped back to control levels. A different temporal response was detected after H_2O_2/Fe^{2+} exposure (Figure 3B): Protein oxidation levels increased after 0.5h administration and immediately decreased to control levels, however, after 8h administration to H_2O_2/Fe^{2+}, protein oxidation levels raised again. This indicated degradation of oxidized proteins and a secondary oxidation of proteins after 8h administration. TBH exposure did not result in increased carbonyl group formation (data not shown).

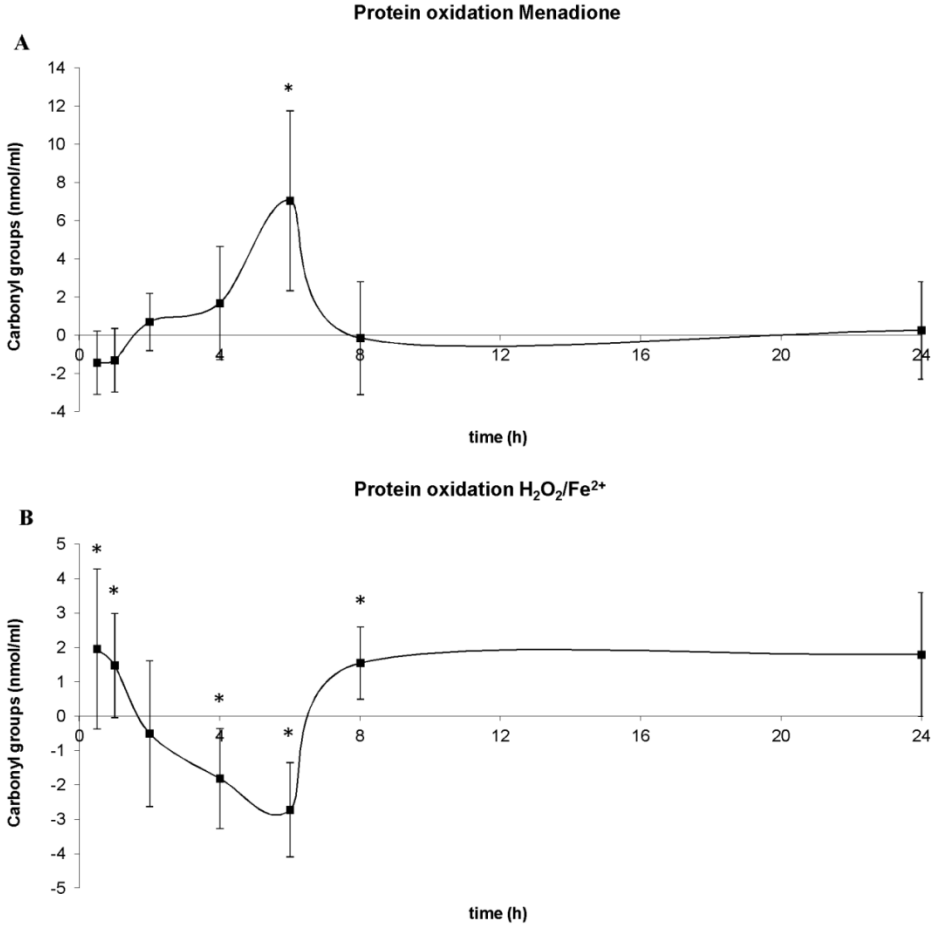

Figure 3 Levels of protein oxidation. Carbonyl groups at 0.5, 1, 2, 4, 6, 8 and 24h after (A) menadione and (B) H_2O_2/Fe^{2+} administration. Exposed values corrected by subtracting control values for each time point. (n=3, $p<0.05$)

Oxidative DNA damage levels

At early time points, incubation with the Fpg enzyme led to a significant increase in DNA strand breaks compared to the standard comet assay, indicating the presence of oxidized purines. This increase was highest after 2h menadione administration (Figure 4A), which is comparable to temporal changes observed for cellular ROS formation which were also highest at 2h. DNA damage was decreased after 4h menadione administration. After 0.5h TBH administration, a significant increase in Fpg-induced DNA damage was observed (Figure 4B), which reached its maximum after 1h. Standard DNA damage followed the same temporal profile. After 2h administration there was no longer any difference between before and after Fpg addition. After H_2O_2/Fe^{2+} administration, Fpg-induced and standard DNA damage was the highest after 0.5h (Figure 4C) as was also observed for cellular ROS formation and protein oxidation, but this decreased after 1h exposure, and disappeared after 2h. Thus, H_2O_2/Fe^{2+} administration had less effect on standard and Fpg-induced DNA damage than the other oxidants, comparable as was observed for other cellular responses.

Oxidant-induced whole genome gene expression

Differentially expressed genes and pathway analysis

Whole genome gene expression analysis over all time points resulted in 4414 significant differentially expressed genes upon menadione administration, 2284 genes after TBH exposure and 483 genes after H_2O_2/Fe^{2+} treatment. In total, 136 genes were commonly modified after exposure to these 3 oxidants (see supplementary data appendix C). Between menadione and TBH there was a larger overlap observed which accounts for 1433 common DEGs (Figure 5A). Among these genes, interleukin 8, 11 and TGF-beta1 were included in the intersection. Nrf2/Keap1 signaling was modified by all oxidants, however, not in a similar matter. menadione and H_2O_2/Fe^{2+} exposure resulted in an early upregulation in Nrf2 expression and a downregulation in Keap1 expression (the latter especially for menadione). Phase 2 genes, such as GCLM, GCLC and HMOX1, were upregulated (more details and visualization in supplementary data appendix D). Hierarchical clustering of these 136 common DEGs showed that early time points (0.5, 1 and 2h) of the 3 oxidants cluster together. However, at later time points (6, 8 and 24h) H_2O_2/Fe^{2+} clustered almost completely apart from menadione and TBH (Figure 5B). For all compounds, an increase of DEGs over time was observed.

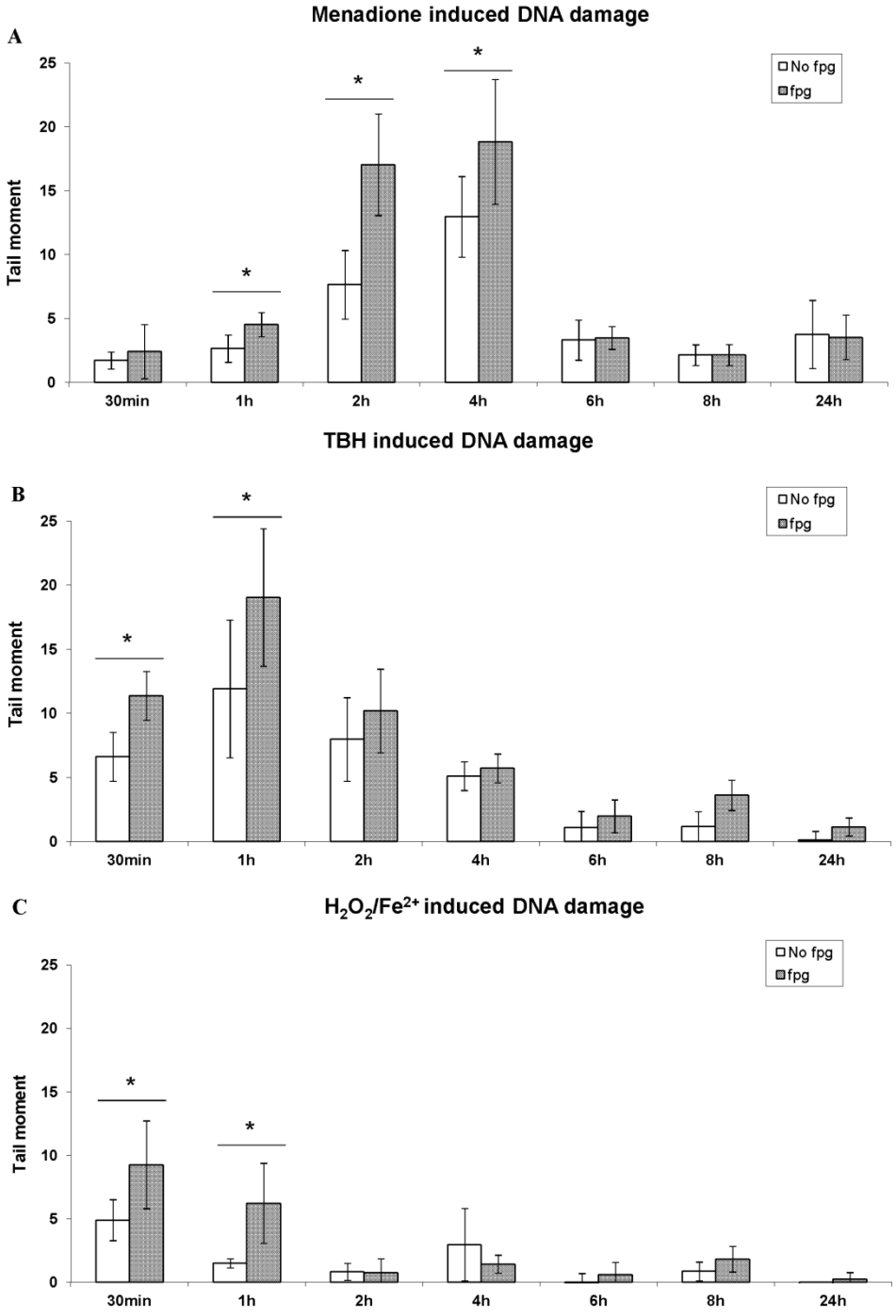

Figure 4 DNA damage using the standard and Fpg comet assay. After Fpg addition indicated the amount of oxidative DNA damage at 0.5, 1, 2, 4, 6, 8, 24h administration to (A) menadione, (B) TBH and (C) H_2O_2/Fe^{2+}. Values were corrected for blank levels. (n=3, $p<0.05$)

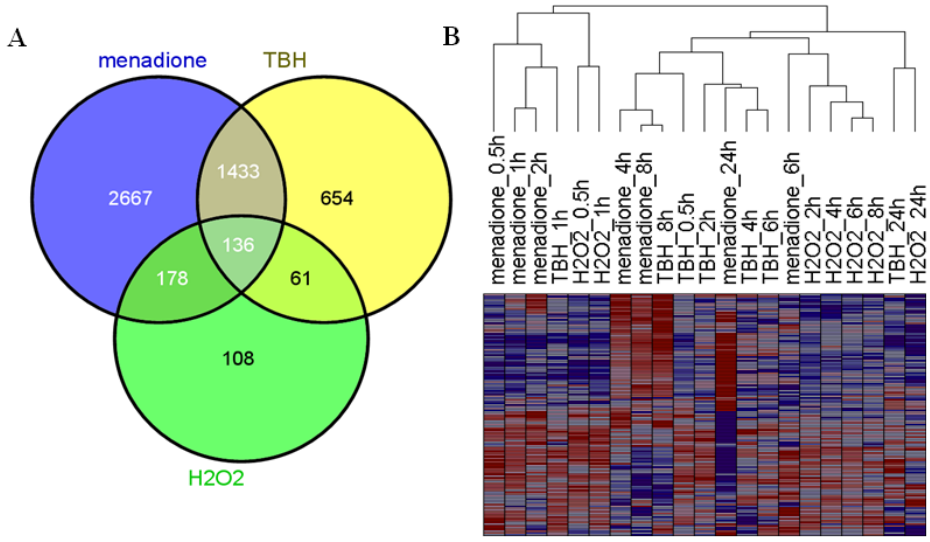

Figure 5 (A) Venn diagram of differentially expressed genes after exposure to menadione, TBH and H_2O_2/Fe^{2+} exposure over all time points showed unique- and overlapping genes. (B) Hierarchical clustering of the genes in the intersection of the three compounds over all time points.

Pathway analysis on common DEGs by the oxidants showed that cell cycle, DNA damage, inflammatory responses, glutathione metabolism and signal transduction pathways such as PKC, Erk and PKA signaling appeared significantly induced (Table 1A). At early time points (0.5, 1, 2 and 4h), glucocorticoid receptor signaling was already found to be affected after exposure to all 3 compounds. At later time points (6, 8 and 24h) TGF-beta receptor signaling was increased by all oxidants. In this gene list of 136 common DEGs, two transcription factors, EGR1 and GCRalpha were identified (Table 1B). Both are involved in immune responses and developmental processes. However, temporal expression profiles of these transcription factors were different for each oxidant. Interestingly, also significant pathways unique for menadione and TBH could be identified. Menadione exposure led to DEGs involved in development whereas TBH exposure led to changes in pathways for lipid biosynthesis and regulation and obesity. Due to the low number of DEGs, no unique pathways were observed for H_2O_2/Fe^{2+} exposure.

Microarray detected changes in expression levels for the oxidative stress responsive genes, SOD1, SOD2, CAT, OGG1, CDKN1A, GPX1 and HMOX1 correlated significantly (Spearman, $R>0.67$, $p<0.05$) with expression levels determined by RT-PCR (see supplementary data appendix B).

Table 1 (A) Significantly regulated pathways based on the common 136 genes. A summary of significantly ($p<0.05$) regulated pathways and related cellular processes as indicated by MetaCore is shown. (B) Significant transcription factor present in the list of the 136 common genes.

A. Pathway analysis	
Pathways and cellular processes	**p value**
Cell cycle and its regulation	
Chromosome condensation in prometaphasis	8.612E-16
Transition and termination of DNA replication	2.335E-13
Start of DNA replication in early S phase	4.688E-11
Immune response	
IFN gamma signaling pathway	1.689E-04
Histamine H1 receptor signaling in immune response	4.340E-05
Inhibitory action of Lipoxins on pro-inflammatory TNF-alpha signaling	5.464E-05
DNA-damage response	
Nucleotide excision repair	5.452E-06
ATM/ATR regulation	1.107E-03
Cell differentiation	
Glucocorticoid receptor signaling	9.308E-04
TGF-beta receptor signaling	8.037E-03
Apoptosis and survival	
BAD phosphorylation	4.668E-03
Anti-apoptotic action of Gastrin	5.256E-03
Regulation of Apoptosis by Mitochondrial Proteins	3.018E-02
Signal transduction	
Activation of PKC via G-Protein coupled receptor	6.421E-05
Erk Interactions: Inhibition of Erk	2.696E-03
PKA signaling	8.490E-03
Oxidative stress regulation	
Glutathione metabolism	1.709E-02
B. Transcription regulation network analysis	
Transcription factor and Gene Ontology terms	**p value**
GCR-alpha	1.100E-62
regulation of transcription from RNA polymerase II promoter	
negative regulation of signal transduction	
regulation of cell communication	
EGR1	9.090E-52
positive regulation of cellular process	
positive regulation of biological process	
response to hormone stimulus	

Co-regulated genes in time-dependent clustering

Using STEM, each gene was assigned to a model profile to which its time series most closely matched based on the correlation coefficient. This tool offers more insights in genes involved in sequential cellular responses to oxidative stress and compound specific time-dependent gene expression. In addition, co-regulated genes in different time clusters can be assigned to different pathways that were identified by MetaCore.

Most pathways were identical for the oxidants but the genes that compromise these pathways showed different temporal expression profiles. Differences in expression levels were especially present at later time points as was also observed in hierarchical clustering of common DEGs (Figure 5B). Menadione administration resulted in 3251 DEGs that were assigned to 5 different time clusters which were linked to cellular processes (Figure 6A). Cluster 1 and 4 showed down-regulated genes that were involved in pathways such as cell cycle, DNA damage and immune responses (complement pathways) (Table 2A). Up-regulated genes were assigned to clusters 2, 3 and 5 and were involved especially in apoptosis and survival processes and immune responses (interleukin pathways). In general, at 6h after administration, gene expressions in these time-dependent gene clusters dropped back to basal levels following a further increase or decrease.

TBH exposure resulted in 5 different time clusters which contained a total of 1795 DEGs (Figure 6B). In 4 out of 5 clusters, a similar temporal profile was observed where especially at 8h after TBH administration a down-regulation of genes present in these clusters was observed. Pathways involved in processes such as cell cycle, DNA damage, apoptosis and survival and lipid metabolism can be assigned to these clusters (Table 2B). Cluster 1 showed up-regulated genes involved in immune responses and development such as glucocorticoid and TGF-beta receptor signaling after 8h administration. After 24h TBH administration, expression levels decreased which was not observed in the other two compounds where up- or down-regulation was maintained after 24h exposure.

Exposure to H_2O_2/Fe^{2+} resulted in 283 DEGs that were assigned to 4 different time clusters (Figure 6C). These clusters showed genes involved in pathways such as cell cycle regulation, development and transcription. Clusters 2 and 4 contained up-regulated genes in these pathways and clusters 1 and 3 contain down-regulated genes in these pathways (Table 2C).

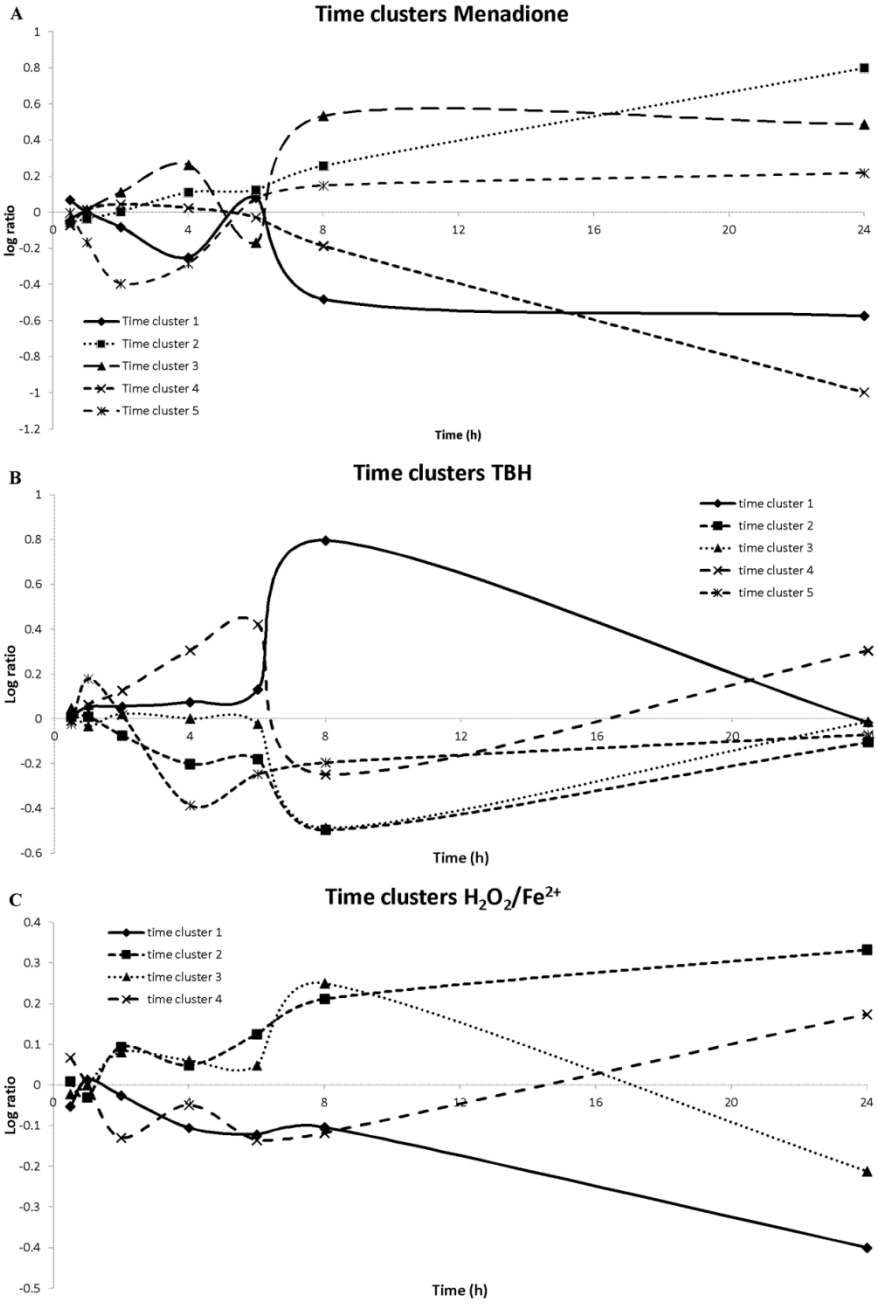

Figure 6 Average expression curves for time dependent gene clusters generated by STEM after exposing HepG2 cells to (A) menadione, (B) TBH and (C) H_2O_2/Fe^{2+} at various time points.

Table 2 Pathways and processes involved in the gene expression cluster profiles. Significantly ($p<0.05$) regulated pathways as indicated by MetaCore of the gene expression profiles found by STEM shown in Figure 6. Tables present a summary of the pathways and cellular processes after (A) menadione, (B) TBH and (C) H_2O_2/Fe^{2+} exposure

A. Pathway analysis in time clustering after menadione exposure

Cluster	Pathways	Processes
1	Chromosome condensation in prometaphasis	Cell cycle
	Start of DNA replication in early S phase	
	Role of Brca1 and Brca2 in DNA repair	DNA damage
	Nucleotide excision repair	
2	Cytoskeleton remodeling	Cytoskeleton remodeling
	BAD phosphorylation	Apoptosis and survival
	Regulation of Apoptosis by Mitochondrial Proteins	
	TGF-beta receptor signaling	Development
3	Oncostatin M signaling via MAPK in human cells	Immune responses
	IL-1 signaling pathway	
	MIF-mediated glucocorticoid regulation	
4	Alternative complement pathway	Immune responses
	Lectin induced complement pathway	
	Classical complement pathway	
5	FAS signaling cascades	Apoptosis and survival
	Caspase cascade	

B. Pathway analysis in time clustering after TBH exposure

Cluster	Pathways	Processes
1	IL-2 activation and signaling pathway	Immune responses
	IL-1 signaling pathway	
	Glucocorticoid receptor signaling	Development
	TGF-beta receptor signaling	
2	Chromosome condensation in prometaphase	Cell cycle
	Initiation of mitosis	
	The metaphase checkpoint	
	Regulation of fatty acid synthase activity	Lipid metablism
	WNT signaling pathway. Part 2	Development
3	Start of DNA replication in early S phase	Cell cycle
	DNA-damage-induced apoptosis	Apoptosis and survival
	p53-dependent apoptosis	
4	Nucleotide excision repair	DNA damage
	Mismatch repair	
	Role of Brca1 and Brca2 in DNA repair	
5	The metaphase checkpoint	Cell cycle
	Spindle assembly and chromosome separation	

Table 2 continued

C. Pathway analysis in time clustering after H_2O_2/Fe^{2+} exposure		
Cluster	**Pathways**	**Processes**
1	Role of APC in cell cycle regulation	Cell cycle
	Start of DNA replication in early S phase	
	Nucleocytoplasmic transport of CDK/Cyclins	
2	Role of APC in cell cycle regulation	Cell cycle
	Regulation of G1/S transition (part 2)	
	P53 signaling pathway	Transcription
3	Role of APC in cell cycle regulation	Cell cycle
	Regulation of G1/S transition (part 2)	
	P53 signaling pathway	Transcription
4	Regulation of G1/S transition (part 2)	Cell cycle
	P53 signaling pathway	Transcription

In addition, similarities and correlations in time-dependent gene expression were evaluated between different experiments using STEM. No correlating clusters were identified between H_2O_2/Fe^{2+} and the other oxidants. However, the overlap between menadione and TBH resulted in 3 correlating time profiles. Genes present in these profiles were especially involved in cell cycle pathways and glutathione metabolism. But most importantly, correlation of time profiles of the 136 common DEGs resulted in only one time profile (Figure 7) that was identical for menadione and TBH. This time profile contained 17 up-regulated genes (Table 3). At least ten of these genes are found to be involved in oxidative stress responses and (hepato)carcinogenesis. Expression of these 17 genes was compared to a previously published negative control for oxidative stress pathways in HepG2 cells, D-mannitol [12]. None of these genes were differentially expressed following D-mannitol administration for 24h.

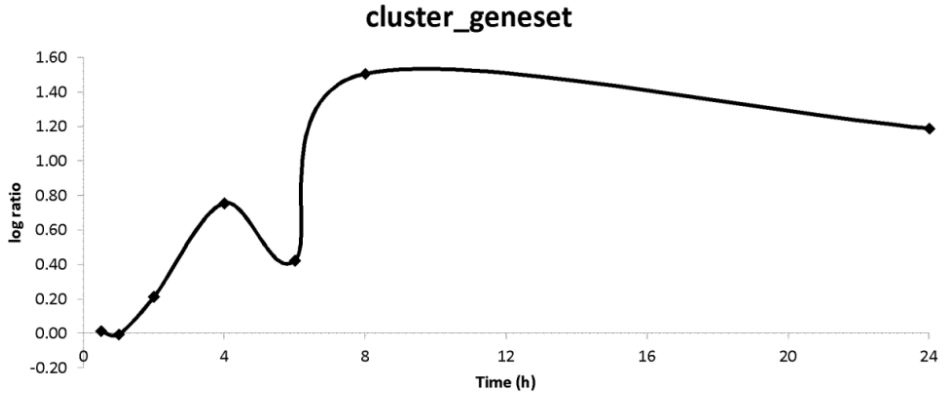

Figure 7 Average expression curve for common time cluster generated by STEM of genes differentially expressed after menadione and TBH administration, present in the intersection.

Table 3 Set of differentially expressed genes in the intersection present in common gene expression time cluster of menadione and TBH exposure.

Gene set of common gene expression time cluster		
Gene	**Full name**	**Biological processes**
SQSTM1	sequestosome 1	NF-κB cascade
BIK	BCL2-interacting killer	Apoptosis
FBXO30	F-box protein 30	Ubiquitin-protein ligase
GCLC	glutamate-cysteine ligase, catalytic subunit	Glutathione biosynthesis
AKR1C2	aldo-keto reductase family 1, member C2	Oxidation-reduction
SRXN1	sulfiredoxin 1	Response to ox. stress
LIF	leukemia inhibitory factor	Development/differentiation
SLC7A11	solute carrier family 7	Response to ox.stress
GCLM	glutamate-cysteine ligase, modifier subunit	Glutathione biosynthesis
AGPAT9	acylglycerol-3-phosphate O-acyltransferase	Lipid metabolism
RRAS2	related RAS viral oncogene homolog 2	Protein signal transduction
ASF1A	anti-silencing function 1 homolog A	DNA repair
RAP1GAP	RAP1 GTPase activating protein	GTP catabolism
AKR1B10	aldo-keto reductase family 1, member B10	Metabolism/catabolism
GCNT3	glucosaminyl (N-acetyl) transferase 3	Carbohydrate metabolism
PTGR1	prostaglandin reductase 1	Metabolism
GSR	glutathione reductase	Glutathione metabolism

Phenotypical anchoring

Correlations between temporal gene expression profiles and changes in endpoints measured (cell cycle distribution, early apoptosis, protein oxidation and oxidative DNA damage) were identified using STEM. Only a small number of genes correlating with functional endpoints following H_2O_2/Fe^{2+} exposure were found (data not shown). However, the annotated functions of these genes are not related to the measured markers for oxidative stress. After TBH administration, time-dependent expression of several genes correlated with changes in the different phases of the cell cycle (G1, S and G2/M) (Table 4). Identified genes were involved in pathways of cell adhesion, chemotaxis and cell cycle. For menadione, phenotypical anchoring revealed that DEGs correlated with almost every endpoint (Table 4). Early apoptosis, which was detected only after menadione administration, correlated with pathways involved in apoptosis and survival, such as the caspase cascade and FAS signaling. In addition for this oxidant, cell cycle pathways were found to correlate with the cells being in S phase and G2/M phase and protein oxidation correlated with pathways involved in protein degradation and anti-oxidant mechanisms like glutathione. Oxidative DNA damage however did not correlate with DNA damage pathways but rather with immune response pathways.

Table 4 Genes that clustered in the same gene expression cluster profiles with measured endpoints such as, apoptosis, cell cycle, protein oxidation and oxidative DNA damage using STEM after (A) menadione and (B) TBH exposure. Significant ($p<0.05$) pathways as indicated by MetaCore.

A. Phenotypical anchoring after menadione administration	
Functional endpoint	**Pathways**
Apoptosis	Apoptosis and survival: BAD phosphorylation, Granzyme B signaling, Caspase cascade, FAS signaling cascades, TNFR1 signaling pathway
G1 phase	-
S phase	Cell cycle: Role of APC in cell cycle regulation, Start of DNA replication in early S phase
G2/M phase	Cell cycle: Nucleocytoplasmic transport of CDK/Cyclins
Protein oxidation	Glutathione metabolism; Proteolysis: Putative SUMO-1 pathway; DNA damage: Role of SUMO in p53 regulation
Oxidative DNA damage	Immune response: IL-3 activation and signaling pathway, IL-5 signaling, IL-2 activation and signaling pathway, ETV3 effect on CSF1-promoted macrophage differentiation
B. Phenotypical anchoring after TBH administration	
Functional endpoint	**Pathways**
Apoptosis	-
G1 phase	DNA damage: Role of Brca1 and Brca2 in DNA repair; Cell cycle: Start of DNA replication in early S phase, The metaphase checkpoint
S phase	G-protein signaling: K-RAS regulation pathway; Cell cycle: Influence of Ras and Rho proteins on G1/S Transition
G2/M phase	-
Protein oxidation	-
Oxidative DNA damage	-

Discussion

The incomplete and fragmentary information of liver cell stress responses to oxygen radicals hampers a reliable prediction of human health risks for chronic liver toxicity of new chemicals and drugs. Although the ROS-generating properties of menadione, TBH and H_2O_2 and its consequences have been extensively investigated in other cell models, so far no studies in hepatoma cells have been published which apply an extensive time series analysis including early time points in order to understand similar and unique sequential cellular and gene expression responses towards different forms of oxygen radicals.

Oxygen radical formation in HepG2 cells after exposure to these three compounds was confirmed using ESR combined with the spin trapping technique. Menadione exposure led to increased and sustained ROS formation compared to

TBH and H_2O_2/Fe^{2+}, which matched observations for oxidative DNA damage and protein oxidation. Loor et al (2010) revealed that menadione induces oxidant stress by producing mainly $O_2^{\bullet-}$. Subsequently, H_2O_2 could be produced by the reduction of these superoxide anion radicals by SOD activity. However, cell death/apoptosis was very low after menadione exposure, which indicates that, also based on Loor et al (2010), especially intracellular $O_2^{\bullet-}$ was formed and this did not result in a lethal amount of H_2O_2. This may also be an explanation for the low effect of menadione on cell cycle distribution measured by flow cytometry. Considering the lower cellular responses to H_2O_2 in comparison to other oxidants in HepG2 cells, also observed in combination with Fe^{2+}, this may be due to high basal catalase activity in these cells, as previously described in the study of [25]. On the other hand, TBH was described as an excellent model of oxidative stress in HepG2 cells [25]. Reductive cleavage of TBH results in methyl and hydroxyl radicals that induce oxidative stress responses such as oxidative DNA damage [26] which was also observed in our data.

At the gene expression level, differences in temporal responses between these three oxidants were also observed. An important observation was that the analysis of time profiles showed similar pathways which were affected after exposure to the three oxidants, but genes involved in these pathways had different expression profiles. This may be the result of differences in time required for reducing the oxidants to form ROS, and the different types of ROS formed by the oxidants.

Affected pathways were especially involved in cell cycle and immune response, and also demonstrated the strongest correlation with markers for oxidative stress after exposure to menadione and TBH. In addition, DNA damage response, apoptosis and oxidative stress regulation were important processes that were up-regulated after exposure to all compounds. However, also significant pathways unique for menadione and TBH could be identified, but were not directly related to oxidative stress mechanisms.

The transcription factors GCRalpha and EGR1 were already up- or downregulated at early time points whereas, TGF-beta receptor signaling was found to be affected after exposure to all 3 compounds at later time points. EGR1, which activates transcription factor GCRalpha was also observed being differentially expressed in our gene expression data. Both these transcription factors are necessary for normal cellular growth, development, metabolism and immune response. Disturbed regulation of EGR1 and GCRalpha can lead to several liver abnormalities [27, 28]. TGF-beta overexpression is correlated with HCC's carcinogenesis, progression, and prognosis [29]. TGF-beta inhibits cell proliferation, but it also promotes tumor cell invasion by inducing epithelial-mesenchymal transition (EMT) [30].

When comparing early and later time points we observed that oxidants clustered together at early time points but more apart at later time points (Figure 5B and Figure 6A-C). This indicates the presence of a more general acute stress response at early time points, which changed into a more compound specific cellular

response later on. For example, exposure to TBH, which is known to induce lipid peroxidation [31], led to changes in lipid metabolism. DNA repair mechanisms such as NER were activated over time after exposure to menadione and TBH, but were not significant differentially expressed after H_2O_2/Fe^{2+} exposure. This was also reflected in double DNA strand breaks and oxidative DNA damage levels compared to the other two compounds. In addition, protein oxidation levels after menadione exposure correlated with glutathione metabolism and protein degradation. However, compound specific cellular responses were especially due to differences in temporal gene expression profiles which can be associated to differences in radical formation and other endpoints measured of each oxidant.

Despite these differences, a similar temporal gene expression profile was identified for menadione and TBH exposure. This cluster consists out of 17 co-expressed genes that are involved in oxidative stress mechanisms and liver carcinogenesis. This specific oxidative stress related gene expression profile can be applied for a better recognition and mechanistic understanding of oxidative stress induced cell damage by chemicals. Specifically, ASF1A has a function in DNA repair [32], where SQSTM1 and BIK, member of the BCL-2 family, are involved in NF-κB signaling [33] and apoptosis [34] respectively GCLC and GCLM are the first rate-limiting enzymes of glutathione synthesis. Another interesting gene in this set is GSR, a central enzyme of cellular antioxidant defense, which reduces oxidized glutathione disulfide (GSSG) to the sulfhydryl form GSH. Furthermore, it is suggested that SRXN1, AKR1B10, SLC7A11 and RRAS2 are involved in tumor growth, progression and metastasis and are linked to HCC [35-38]. Since these findings were established using HepG2 cells, validation in other (primary) cell lines and/or other compounds would be of interest.

In conclusion, using an extensive time series and phenotypical anchoring, we were able to identify unique and similar genes and pathways affected after generation of different types of oxygen radicals. Furthermore, we observed a more general acute stress response at early time points, which changed into a more compound-specific cellular response later on. Most importantly, a gene set with a similar expression profile over time was found and this could serve as an oxidative stress model to investigate chemicals (drugs, potential (non-gentoxic) hepatocarcinogens) for their oxidative stress capacities. This strongly highlights that studying time-dependent cellular effects is very helpful for developing an in depth understanding of oxidative stress induced cellular defense mechanisms and responses in cell models.

Supplementary data

You can download all supplementary data using following URL:
https://mega.co.nz/#F!7slk1ZaY!guSiYIR4SerSyanPmArzag

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CHAPTER 3

Cell line-specific oxidative stress in cellular toxicity: A toxicogenomics-based comparison between liver and colon cell models

Toxicology in vitro 2015

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Abstract

Imbalance between high reactive oxygen species formation and antioxidant capacity in the colon and liver has been linked to increased cancer risk. However, knowledge about possible cell line-specific oxidative stress-mechanisms is limited. To explore this further, gene expression data from a human liver and colon cell line (HepG2/Caco-2), both exposed to menadione and H_2O_2 at six time points (0.5-1-2-4-8 and 24h) were compared in association with cell cycle distribution. In total, 3164 unique- and 1827 common genes were identified between HepG2 and Caco-2 cells. Despite the higher number of unique genes, most oxidative stress-related genes such as CAT, OGG1, NRF2, NF-κB, GCLC, HMOX1 and GSR were differentially expressed in both cell lines. However, cell-specific regulation of genes such as KEAP1 and GCLM, or of the EMT pathway, which are of pathophysiological importance, indicates that oxidative stress induces different transcriptional effects and outcomes in the two selected cell lines. In addition, expression levels and/or -direction of common genes were often different in HepG2 and Caco-2 cells, and this led to very diverse downstream effects as confirmed by correlating pathways to cell cycle changes. Altogether, this work contributes to obtaining a better molecular understanding of cell line-specific toxicity upon exposure to oxidative stress-inducing compounds.

Introduction

Oxidative stress may occur in almost any tissue and is believed to play an important role in carcinogenesis. To sustain a proper regulation of biological processes, a physiological balance between the formation of reactive oxygen species (ROS) and the antioxidant network is essential [1, 2]. Small amounts of ROS produced under normal physiological conditions have a protective role in the cell, however, overproduction may lead to accumulation in the intracellular environment resulting in oxidative stress, which subsequently leads to damage to various cell structures [3]. Spontaneous mutations that are then induced by oxidative stress may lead to carcinogenesis [4], and various cancers have been found to be in a constant state of oxidative stress, which suggests a role for oxidative stress in cancer promotion as well [5]. As portals of entry for xenobiotics, the liver and the gastrointestinal tract are continuously exposed to multiple chemicals, and as such are prone to oxidative damage induced by different types of oxidative compounds. As a consequence, the imbalance between ROS formation and antioxidant capacity in the colon and liver has been linked to increased cancer risk [6]. ROS-induced mechanisms have actually been related to different chronic liver diseases and hepatocellular carcinoma (HCC), and are induced by various risk factors for liver cancer such as hepatitis B and C or aflatoxin-B1 [7]. In addition, patients with inflammatory bowel diseases, accompanied by oxidative stress [8], are at increased risk for developing colorectal cancer [9].

Both liver and colon are equipped with defense mechanisms to limit oxidative stress induced damage. The nuclear factor E2-related factor 2 (NRF2) is a key regulator in the oxidative stress response and is expressed in a wide number of tissues, including liver and colon [10]. Under non-stressful physiological condition, NRF2 is kept in the cytosol by KEAP1 [11]. Oxidative stress may modify KEAP1 directly to cause their dissociation and consequently, NRF2 can escape proteosomal degradation and translocates to the nucleus to activate the antioxidant response element (ARE) which facilitates the transcriptional machinery in protecting the cell against oxidative stress [12]. Glutathione biosynthesis is regulated by this cascade which activates its rate-limiting enzymes GCLC and GCLM and is believed to be involved in multiple liver diseases, as well as in chemo-resistance in HCC [13]. Furthermore, nuclear factor-κB (NF-κB) is translocated to the nucleus after induction by oxidative stress to activate genes involved in inflammation and immune responses, apoptosis and proliferation [14].

When these first line defense mechanisms fail in preventing oxidative stress-induced cellular damage, other processes such as DNA damage repair, cell cycle arrest or programmed cell death can be activated to prevent the formation of fixed mutations. However, when ROS levels are excessively elevated in cells, oxidative stress and consequently chronic inflammation will be induced. Attracted immune cells will constantly generate new ROS resulting in chronic oxidative stress

which will induce fixed DNA mutations and will contribute to carcinogenesis by activating oncogenes and/or inactivating tumor suppressor genes [15, 16].

Where oxidative stress-related mechanisms described so far, seem to be quite generic, cell type-specific signaling pathways in cellular damage and carcinogenesis-induced by oxidative stress may underlie risks for chronic inflammation and carcinogenesis in particular target organs. For contributing to cancer prevention and treatment of tissue-specific cancers, it thus is of primary importance to investigate such cell type-specific differences at the molecular level. Since oxidative stress-related effects will differ in time, examining and comparing temporal changes in different cell types is of additional relevance. In previous *in vitro* studies, these oxidative stress-related mechanisms in response to different oxidants were extensively investigated using such a time series gene expression approach [17, 18]. These cellular models readily allow for time-dependent analysis of whole genome gene expression, so here, we compare oxidant-induced gene expression changes and cell cycle distribution data from these previous performed *in vitro* studies in a human hepatoma cell line (HepG2 cells) and a human colon adenocarcinoma cell line (Caco-2 cells). Since both these cell lines respond to oxidative stress in activating antioxidant machineries [17, 18], are able to carry out biotransformation of xenobiotics and are permeable for different types of compounds [19, 20], these cells are a convenient and reproducible *in vitro* alternative for *in vivo* toxicity testing. Both cell lines were exposed to menadione, a polycyclic aromatic quinone generating superoxide after redox cycling mediated by quinone oxidoreductase (NQO1), and H_2O_2 which can oxidize transition metals using the Fenton reaction to create hydroxyl radicals and is metabolized by catalase. Using a range of bioinformatics tools, unique and common genes/pathways will be identified as well as temporal expression profiles of differentially expressed genes (DEGs) investigated. In particular, oxidative stress related pathways such as the NRF2/KEAP1 pathway will be examined, as well as the role of NF-κB in both cell types including different down-stream effects which may be associated with cell type-specific pathologies.

Material and methods

Cell culture

HepG2 and Caco-2 cells (ATCC, LGC logistics, UK) were cultured in 6-well plates as previously described [17, 18]. When cells were 80% confluent, the medium was replaced with medium containing 100µM menadione in both cell lines (Sigma-Aldrich, Zwijndrecht, The Netherlands) or 50µM H_2O_2/Fe^{2+} in HepG2 cells and 20µM H_2O_2 in Caco-2 cells (VWR int, UK). These non-cytotoxic concentrations were selected as previously described using MTT and ESR spectroscopy [17, 18]. An exposure time series was applied in both cell lines (0.5, 1, 2, 4, 8 and 24h) and

time-matched control cells (only medium) were treated in an identical manner without addition of oxidants.

Cell cycle distribution

Analyses of cell cycle profiles were performed as previously described [21]. Cells were stained with propidium iodide and cell cycle profiles were analyzed using ModFit LT for Mac (version 2.0).

Quantitative PCR and whole genome gene expression

First RNA was extracted using QIAZOL in combination with MiRNeasy mini kits (Qiagen, Westburg, The Netherlands) and quality was assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Amstelveen, The Netherlands) as previously described [18].

Quantitative PCR was performed in biological duplicates for both treated and untreated HepG2/Caco-2 cells and calculated as previously reported [21, 22] (n=2). RT-PCR was run on the MyiQ Single-Color RT-PCR Detection System (Bio-Rad Laboratories). Forward and reverse primers of Beta-Actin (used as reference), HMOX1, BCL2, GCLC, MAFG and NQO1 can be found in supplementary data 1.

cRNA from treated and untreated HepG2 cells was prepared using Affymetrix synthesis and labeling kits as described before (Affymetrix, Santa Clara, CA) [19]. cRNA targets of control and exposed were individually hybridized on high-density oligonucleotide genetitan chips (Affymetrix Human Genome U133 Plus PM GeneTitan 24 arrays) as previously described [18]. Two oxidant exposures and time matched control samples during six different time points in biological triplicate (n=3) resulted in a total of 72 single-color arrays.

Exposed and time matched control RNA samples from Caco-2 cells were two-color labeled and hybridized on the same array and scanned according to the manual for G4110B 22K/G4112F 44K Agilent Human Oligo Microarray (Agilent Technologies, Santa Clara, CA) as previously described [17]. Two oxidant exposures and time matched control samples during six different time points in biological duplicates and technical duplicates (dye swap) resulted in a total of 48 two-color arrays.

Re-annotation and normalization

In HepG2 cells, data from 72 arrays were obtained, and Robust Multi-array Average (RMA) normalized and re-annotated to custom CDF files using the array analysis tool (http://arrayanalysis.org/). In Caco-2 cell, images of 48 hybridizations were processed with ImaGene 6.0 software (BioDiscovery Inc., Los Angeles, CA) to quantify spot signals and normalized in GeneSight software version 4.1.6 (BioDiscovery Inc.) as previously described [17]. The data discussed in this publication have been deposited in NCBI's gene expression omnibus [23, 24]:
GSE39291: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE39291,
GSE15327: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE15327

Data filtering and analysis

Normalized datasets of both array platforms were compared, and only genes found on both types of array platforms (Agilent and Affymetrix) were selected. The intensities of the filtered data sets were log2 transformed, and subsequently, log ratios of treated versus controls were calculated. Differentially expressed genes (DEGs) for each experimental group were selected by using criteria that were described before [18]: (1) log ratio of <-0.26 or >0.26, (2) same direction for all replicates, (3) intensity of log2 values >6 for at least 2 out of 3 replicates, and (4) $p<0.05$. A gene was defined as being a DEG when it met all criteria, as described in material and methods, for at least one time point. The union of DEGs found after menadione and H_2O_2 exposure was used for further analysis (overlapping and unique DEGs between menadione and H_2O_2) and average log ratios were calculated.

Data clustering

DEGs induced by all compounds were clustered using the Hierarchical Clustering Analysis (HCA; Pearson correlation, Euclidean distance, pair wise complete distance) in GenePattern v.3.2.1 (http://genepattern.broadinstitute.org).

Short Time-series Expression Miner (STEM)

For identification of genes co-regulated time-dependently and clustering with the markers for oxidative stress, the software tool ‘‘Short Time-series Expression Miner’’ (STEM, version 1.1.2b; http://www.cs.cmu.edu/~jernst/stem/) [25] was used. Criteria used were described before [17]. For correlation analysis, data from cell cycle distribution levels were transformed into log 2 base ratios.

Dynamic Time warping (DTW)

In order to explore cases where the time courses in the two organs were similar but not simultaneous, the DEGs were aligned using the matched functionality in dtw4omics [26], an R package for dynamic time warping. To select those genes with significantly matching time courses a False Discovery Rate (FDR) of 5% was applied.

Pathway analysis

MetaCore (GeneGo, San Diego, CA) was used to identify and visualize the involvement of the differentially expressed genes and transcription factors in the biological processes that may be affected at the level of pathways, by selecting significant pathways with a p value < 0.05 and false discovery rate (FDR) of <5%.

Results

Comparison of oxidative stress-induced global gene expression changes in HepG2 and Caco-2 cells

Time-dependent exposures to menadione and H_2O_2, in HepG2 and Caco-2 cells resulted in 72 and 48 raw transcriptomics data sets respectively conducted on two different array platforms as described in material and methods. In the MicroArray Quality Control (MAQC) project, intra- and interplatform data comparison was intensively studied and showed a high level of concordance and reproducibility within and between different platforms [27, 28]. Raw expression values generated on different platforms cannot be directly compared because unique labeling methods which result in variable signals for probes that hybridize to the same target. Therefore, results obtained from both platforms were re-annotated, normalized and filtered in a similar manner and log ratios were calculated to obtain information in terms of differentially expressed genes (DEGs). In this way, we were certain that the following discussed differences between HepG2 and Caco-2 cells were cell type-specific and not platform-specific. In addition, we analyzed basal gene expression in control samples and, since basal gene expression and active pathways, such as cell cycle, apoptotic and developmental processes, were primarily similar between HepG2 and Caco-2 cells, these differences/similarities resulted from a different response to oxidant exposure and cannot be assigned to basal gene expression differences.

Single oxidant comparison

HepG2 cells and Caco-2 cells were exposed to previously selected non-cytotoxic concentrations [17, 18] of two different oxidants, each producing a different oxygen radical, mainly superoxide anion produced by menadione, and hydroxyl radicals produced by H_2O_2. Transcriptomics data of both HepG2 and Caco-2 cells showed the expression of NQO1, responsible for reduction of menadione as well as catalase activity to metabolize H_2O_2 [29]. In addition, previously obtained ESR data [17, 18] showed the formation of comparable amounts of oxygen radicals in Caco-2 and HepG2 cells following exposure to menadione and H_2O_2. These results indicate that both cell lines were able to metabolize both compounds to produce ROS.

When comparing menadione exposure in both cell types, 1023 DEGs overlapped, 1351 DEGs were unique in HepG2 cells and 1369 DEGs were unique in Caco-2 cells. H_2O_2 exposure resulted in 159 overlapping DEGs between cell types, 111 unique DEGs in HepG2 cells, and 3127 unique DEGs in Caco-2 cells. Both overlapping and unique genes are involved in oxidative stress and DNA damage responses. To obtain a better understanding of global oxidative stress responses instead of specific effects of different reactive oxygen species, data sets of menadione and H_2O_2 were combined using the average expression of each

gene in HepG2 and Caco-2 cells. Both overlapping and unique DEGs for both oxidants were included in further analysis.

Global oxidative stress-induced changes in HepG2 and Caco-2 cells

When comparing gene expression changes induced by oxidative stress in both cell types, a total of 1827 common DEGs and 637 unique DEGs in HepG2 and 2527 DEGs in Caco-2 cells (Figure 1A) were found. The number of DEGs, either up- or downregulated, was lower in HepG2 cells at all measured time points. In addition, clustering of these overlapping genes showed that expression levels at early time points in Caco-2 cells (0.5 and 1h) were more similar to expression levels at later time points in HepG2 cells (2 and 4h) (Figure 1B). This shift was also observed in expression levels in HepG2 and Caco-2 cells at later time points and may indicate that comparable gene expression changes seem to appear earlier in Caco-2 cells.

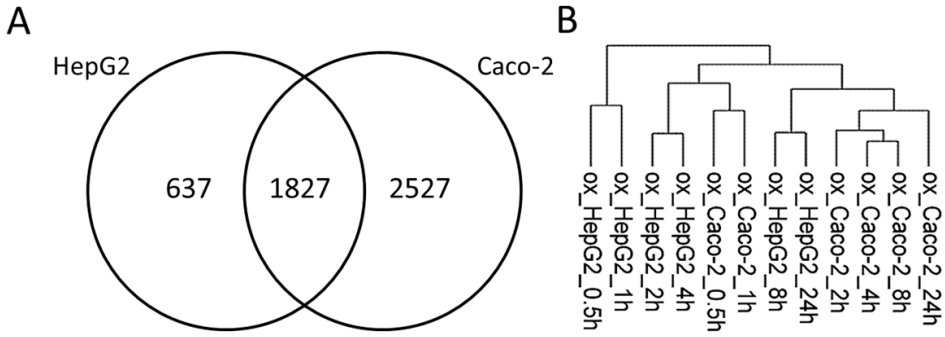

Figure 1 (A) Venn diagram of differentially expressed genes after exposure to oxygen radicals over all time points showed unique- and overlapping genes between HepG2 and Caco-2 cells. (B) Hierarchical clustering of overlapping genes between HepG2 and Caco-2 cells over all time points. Expression levels of genes at early time points in Caco-2 cells (0.5 and 1h) clustered together with later time points in HepG2 (2 and 4h). Later time points in HepG2 (8 and 24h) clustered together with expression levels of genes during 2-24h exposure.

Table 1 (A) Significantly regulated pathways based on the overlapping genes, (B) unique genes in HepG2 cells and (C) unique genes in Caco-2 cells. A summary of significantly ($p<0.05$) regulated pathways and related cellular processes as indicated by MetaCore is shown.

A. Overlapping pathways	
Pathways and cellular processes	**p value**
Cell cycle and its regulation	
Chromosome condensation in prometaphasis	5.487E-12
Transition and termination of DNA replication	1.484E-08
Initiation of mitosis	3.383E-08
DNA-damage response	
ATM/ATR regulation of G1/S checkpoint	1.063E-08
Nucleotide excision repair	1.457E-04
Immune Responses	
IFN gamma signaling pathway	1.683E-05
Signaling pathway mediated by IL-6 and IL-1	1.638E-04
Apoptosis and survival	
p53-dependent apoptosis	7.108E-04
Role of PKR in stress-induced apoptosis	1.063E-03
Development	
WNT signaling pathway. Part 2	1.332E-05
TGF-beta receptor signaling	1.511E-04
Signal transduction	
AKT signaling	1.540E-04
JNK pathway	2.366E-03
PTEN pathway	1.483E-02
B. Unique pathways in HepG2 cells	
Pathways and cellular processes	**p value**
Transcription	
Role of HP1 family in transcriptional silencing	5.631E-06
TGF-beta-dependent induction of EMT via RhoA, PI3K and ILK	4.517E-04
Cytoskeletal remodeling	
CDC42 in cellular processes	9.228E-05
Cell cycle	
The metaphase checkpoint	1.139E-04
Start of DNA replication in early S phase	5.909E-04
C. Unique pathways in Caco-2 cells	
Pathways and cellular processes	**p value**
Protein folding and maturation	
POMC processing	1.368E-11
Bradykinin / Kallidin maturation	8.354E-04
Cytoskeleton remodeling	
TGF, WNT and cytoskeletal remodeling	3.154E-09
Role of PKA in cytoskeleton reorganization	1.091E-05
Others	
Oxidative phosphorylation	7.302E-10
Some pathways of EMT in cancer cells	3.214E-07

Oxidative stress affected genes and pathways in HepG2 and Caco-2 cells

The 1827 genes differentially expressed in both HepG2 and Caco-2 cells included known oxidative stress-related genes such as CAT, OGG1, NRF2, NF-κB, GCLC, AHR, HMOX1, GSR, GSTA1-4 and MSH6. Interestingly, KEAP1, inhibitor of NRF2, was upregulated by oxidative stress only in HepG2 cells, as were Phase II gene GCLM and tumor suppressor gene PTEN which were up- and downregulated respectively. Unique genes in Caco-2 cells were among others involved in apoptosis and cell cycle, such as BAD, BCL2 and 3, PARP1 which were all downregulated, and CDKN1C and CDNK2D which were both upregulated. Also HMOX2 was significantly upregulated only in Caco-2 cells.

The 1827 overlapping genes were involved in a total of 75 significant pathways. Unique genes in HepG2 and Caco-2 genes were involved in 6 and 171 significant pathways respectively; the most significant pathways are shown in Table 1. This difference in number of pathways is due to a higher number of unique DEGs found in Caco-2 cells exposed to oxidative stress, which were consequently involved in more pathways. Overlapping pathways were especially involved in cell cycle processes, DNA damage and immune responses. Pathways most significantly affected and included in this overlap were chromosome condensation, nucleotide excision repair, WNT-, NOTCH1-, TGF-beta receptor- and p53 signaling. Pathways containing unique DEGs found in HepG2 cells are involved in calcium signaling, immune responses such as IL-7 signaling in B and T lymphocytes, and metabolic processes, while pathways comprising unique DEGs in HepG2 cells, are transcriptional silencing by HP1, induction of epithelial-mesenchymal transition (EMT) by TGF-beta and cytoskeletal remodeling. Pathways containing unique DEGs found in Caco-2 cells were especially involved in protein folding (POMC processing), oxidative phosphorylation and other pathways of EMT.

Transcriptional regulation and downstream effects in HepG2 and Caco-2 cells induced by oxidative stress

32 commonly regulated transcription factors in HepG2 and Caco-2 cells were identified, including oxidative stress-related factors such as NF-κB, NRF2 and EGR1 (Table 2). In addition, AHR and MYC were also affected in both cell types, however, both these transcription factors were upregulated in HepG2 cells, and downregulated in Caco-2 cells.

Oxidative stress is known to activate important pathways such as the anti-oxidant NRF2/KEAP1 pathway and inflammatory pathways regulated by NF-κB. Therefore, an oxidative stress-regulated effect on these pathways in both cell types is visualized in Figure 2. NRF2 is upregulated in both cell types (Figure 2A and B), however, stronger induced in Caco-2 cells where Keap1 is not differentially expressed (Figure 2B).

Table 2 Common transcription factors found in the 1827 overlapping DEGs of HepG2 and Caco-2 cells and their overall expression direction over time

Common transcription factors in HepG2 and Caco-2 cells		Up(↑)/down(↓) regulated	
TF	**Full name**	**HepG2**	**Caco-2**
AHR	aryl hydrocarbon receptor	↑	↓
AP3M1	adaptor-related protein complex 3, mu 1 subunit	↓	↑
ATF3	activating transcription factor 3	↑	↑
CEBPA	CCAAT/enhancer binding protein (C/EBP), alpha	↓	↓
CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	↑	↓
CEBPD	CCAAT/enhancer binding protein (C/EBP), delta	↓	↓
CITED2	Cbp/p300-interacting transactivator carboxy-terminal domain 2	↓	↓
E2F3	E2F transcription factor 3	↓	↓
EGR1	early growth response 1	↑	↓
ETS2	v-ets erythroblastosis virus E26 oncogene homolog 2	↑	↓
FOSL1	FOS-like antigen 1	↑	↑
FOXA1	forkhead box A1	↓	↓
FOXA2	forkhead box A2	↓	↓
FOXA3	forkhead box A3	↓	↑
FOXM1	forkhead box M1	↓	↓
JUN	jun oncogene	↑	↑
JUNB	jun B proto-oncogene	↑	↑
JUND	jun D proto-oncogene	↑	↓
KLF6	Kruppel-like factor 6	↑	↓
MAFG	v-maf musculoaponeurotic fibrosarcoma oncogene homolog G	↑	↓
MAFK	v-maf musculoaponeurotic fibrosarcoma oncogene homolog K	↑	↑
MYC	v-myc myelocytomatosis viral oncogene homolog	↑	↓
NFE2L2	nuclear factor (erythroid-derived 2)-like 2	↑	↑
NF-KB	nuclear factor kappa-light-chain-enhancer of activated B cells	↓	↓
NR2F2	nuclear receptor subfamily 2, group F, member 2	↓	↓
NR3C1	nuclear receptor subfamily 3, group C, member 1	↑	↓
NR4A1	nuclear receptor subfamily 4, group A, member 1	↑	↓
SMAD3	SMAD family member 3	↑	↓
SREBF1	sterol regulatory element binding transcription factor 1	↓	↑
STAT6	signal transducer and activator of transcription 6	↑	↑
TEAD4	TEA domain family member 4	↑	↑
XBP1	X-box binding protein 1	↓	↓

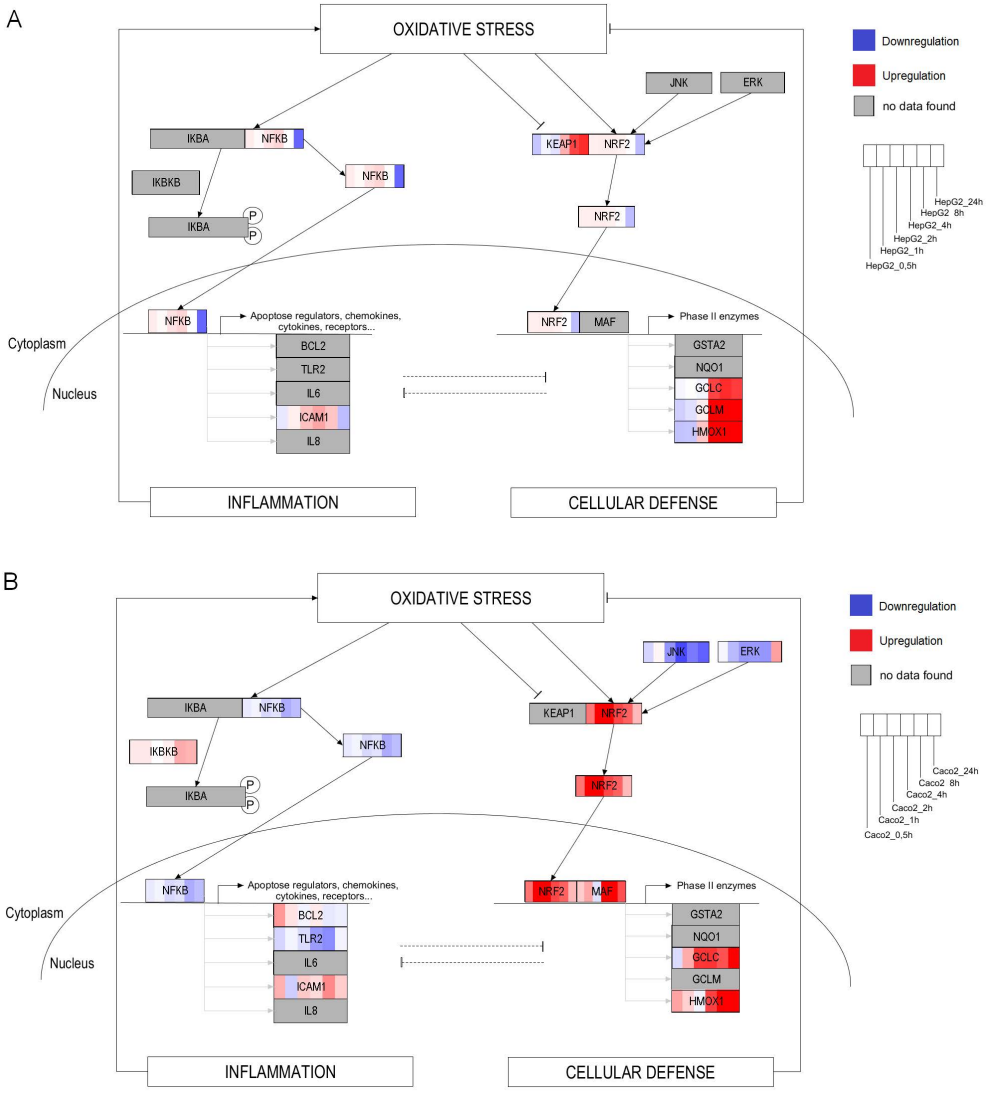

Figure 2 Schematic representation of expression levels of genes involved in the NRF2/KEAP1 and NF-κB pathway activated by oxidative stress in (A) HepG2 cells and (B) Caco-2 cells. Nrf2 in the cytoplasm is bound to its repressor molecule, Keap1. Oxidative stress (as well as Nrf2 phosphorylation by protein kinases) causes dissociation of Nrf2-Keap1 complex, followed by nuclear translocation of Nrf2. Within the nucleus, Nrf2 promotes transcriptional activation of antioxidant enzymes by binding to the antioxidant responsive elements (ARE) in the promoter regions of the target genes. Simultaneously via phosphorylation of the repressor molecule IκB, oxidative stress can cause activation of NF-κB leading to transcriptional activation of genes encoding inflammatory cytokine, chemokines, receptors and apoptotic genes. There is evidence that the Nrf2 and NF-κB pathways exert mutual inhibitory influence on one another. The pathway was constructed by PathVisio using pathway information of Wikipathways. A colored version of this figure can be found online (red= upregulated, blue= downregulated).

In addition, the downstream Phase II gene, GCLM, is also not differentially expressed in Caco-2 cells. Upstream activators of NRF2, ERK and JNK, are significantly downregulated in Caco-2 cells and were not affected in HepG2 cells (Figure 2A and B). Interestingly, NF-κB was significantly downregulated at 24h in both cell types (Figure 2). However, downstream genes of NF-κB, such as BCL2, TLR2 and ICAM1, were differently expressed in Caco-2, while only expression of ICAM1 was affected in HepG2 cells.

Validation using quantitative PCR

Micro-array detected changes in expression levels for the oxidative stress responsive genes, SOD1, SOD2, CAT and P21 were validated as published before [17, 18]. Additionally, expression levels of HMOX1 (R>0.9), NQO1 (R>0.9), BCL2 (R>0.9), GCLC (R>0.9) and MAFG (R>0.78), observed by micro-array, highly correlated with expression levels determined by qPCR (Supplementary data 2).

Comparison of phenotypical anchoring between transcriptomics and cell cycle changes in HepG2 and Caco-2 cells

To obtain more information how HepG2 and Caco-2 cells differ in functional responses towards oxidative stress, data from cell cycle distribution by oxidant exposure was [17, 18] correlated to gene expression changes over time using STEM analysis.

Correlations between changes in the G1, S and G2 phase of the cell cycle induced by oxidative stress in HepG2 and Caco-2 cells, and transcriptomics changes provided a significant number of genes that correlated to the S phase in both cell types, and to the G1 phase in Caco-2 cells and the G2 phase in HepG2 cells. However, gene expression changes correlating to these cell cycle phases were entirely different between HepG2 and Caco-2 cells. Genes correlating to cell cycle changes were mainly involved in cell cycle processes, immune responses, transcription and metabolism of different amino acids and apoptosis and survival pathways as shown in Table 3. In more detail, the percentages of HepG2 cells present in the S phase during oxidative stress decreased after 24h (Figure 3) and correlated with downregulated expression of CDK1, CDK3 and CCNB2, all well described genes in the cell cycle machinery. These correlating genes were mainly involved in pathways such as the initiation of mitosis, and chromosome condensation in prometaphasis (Table 3). On the other hand, the percentage of Caco-2 cells during this stage of the cell cycle was increased (Figure 3) and correlated significantly to upregulated genes involved in IL-1 signaling and transcriptional regulation of aminoacid metabolism (Table 3). In addition, the percentage of Caco-2 cells in the G1 phase was reduced after 24h indicating an S phase arrest following oxidative stress (Figure 3). This change in cell cycle distribution correlated with downregulated genes involved in leucin and valine metabolism, but also chromosome condensation as indicated in Table 3.

Table 3 Genes that clustered in the same gene expression cluster profiles with cell cycle changes induced by oxidative stress (as visualized in figure 3) in (A) HepG2 cells and (B) Caco-2 cells using STEM. Significant ($p<0.05$) pathways as indicated by MetaCore.

A. Phenotypical anchoring in HepG2 cells

Phenotypical endpoint	Pathways
G1 phase	-
S phase	Cell cycle: Chromosome condensation in prometaphase, Sister chromatid cohesion, Initiation of mitosis, Role of APC in cell cycle regulation DNA damage response: ATM / ATR regulation of G2 / M checkpoint
G2/M phase	Developmental processes: Activation of ERK by Kappa-type opioid receptor Apoptosis and survival processes: Ceramides signaling pathway, TNF-alpha-induced Caspase-8 signaling

B. Phenotypical anchoring in Caco-2 cells

Phenotypical endpoint	Pathways
G1 phase	Metabolism: leucine, valine and lysine metabolism Cell cycle: Chromosome condensation in prometaphase
S phase	Immune response: IL-1 signaling pathway Transcription: regulation of aminoacid metabolism
G2/M phase	-

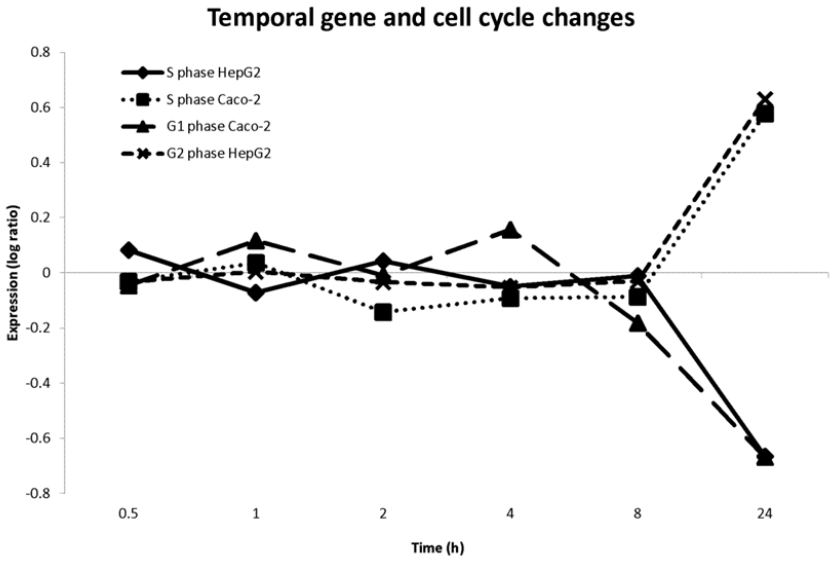

Figure 3 Cell cycle changes over time in log ratio with significant correlating genes following that profile over time. Expression curves were generated by STEM and showed genes correlating to cell cycle endpoints S phase in HepG2 and Caco-2 cells, G1 phase in Caco-2 cells and G2 phase in HepG2 cells after 0.5, 1, 2, 4, 8 and 24 hours oxidative stress.

The increased percentage of HepG2 cells in G2 phase following oxidative stress (Figure 3) correlated to upregulated genes involved in programmed cell death by ceramides and ERK activation (Table 3).

These results indicated that cell cycles of HepG2 and Caco-2 cells were differently regulated in response to oxidative stress. This was mainly observed by the fact that pathways correlating to cell cycle changes induced by oxidative stress were entirely different between HepG2 and Caco-2 cells.

Temporal gene expression analysis between HepG2 and Caco-2 cells following oxidative stress

Using STEM, each gene was assigned to the model profile which its temporal expression profile most closely matched, based on the correlation coefficient. In this study, oxidative stress in HepG2 cells resulted in 2464 DEGs that were assigned to 8 significant time profiles, whereas exposure in Caco-2 cells resulted in 4354 DEGs that were assigned to 11 significant time profiles. Five identical time profiles between both cell models were identified as visualized in Figure 4. This means that genes assigned to these time clusters follow a similar expression profile over time in both cell lines.

Three of these clusters (1, 2 and 5) contain genes that were downregulated over time, and two clusters (3 and 4) contain only genes which were upregulated over time. Interestingly, the oxidative stress-related genes CAT, HMOX1 and GCLC follow a similar expression profile over time in both cell types. Expression of CAT is downregulated over time (Figure 4, cluster 2), whereas HMOX1 and GCLC are similar upregulated over time as also observed in Figure 2 (Figure 4, cluster 4). In contrast, BIK and JUN were upregulated over time in HepG2 cells (Figure 4, cluster 4) and at the same time downregulated in Caco-2 cells (Figure 4, cluster 5). In addition, expression of transcription factors NF-κB and NRF2 decreases over time in both cell types, however following a different expression profile during oxidative stress. Pathway analysis of genes that were assigned to these clusters (Figure 4) is presented in table 4. Overlapping pathways such as NER in DNA damage, WNT signaling and chromosome condensation in prometaphase (Table 4A) were found to contain genes that were downregulated over time. In contrast, genes involved in overlapping pathways such as IL13 signaling and MIF-induced cell adhesion were found to be upregulated over time.

As previously mentioned, we found that oxidative stress results in more cell-specific than commonly differentially expressed genes over time and consequently led to more unique than shared time related pathways. For example, at the moment that cell cycle and DNA damage pathways are regulated in HepG2 cells, immune and apoptotic pathways are regulated in Caco-2 cells (Table 4B-C).

In addition, dynamic time warping (DTW) was used to find comparable changes in gene expression that do not occur simultaneously [26]. Only 58 significant associations were found between HepG2 and Caco-2 cells, what again indicates more differences than similarities between both these cell types. 16 genes were involved in responses to chemical stimuli and 3 genes in glutathione transferase. Oncogene ERBB2 was found to have a similar expression profile in HepG2 and Caco-2 cell with a delay in HepG2 cells. GSTA4, a gene involved in glutathione transferase showed also a similar expression profile in HepG2 and Caco-2 cells, however, this profile had a delay in Caco-2 cells. The opposite was found for MGST2 which peaked earlier in Caco-2 cells, resulting in different effects within the glutathione transferase pathway in HepG2 and Caco-2 cells, which will lead to differences in the defense to cellular oxidative stress by glutathione.

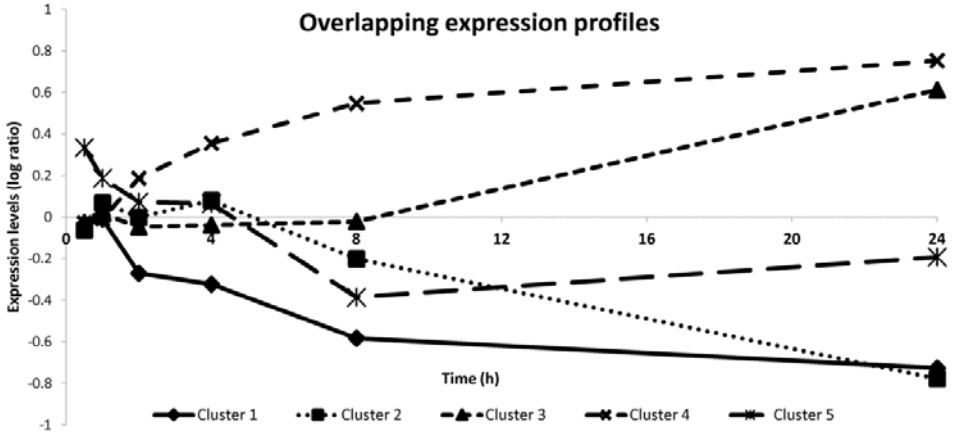

Figure 4 Average overlapping expression curves for time dependent gene clusters generated by STEM after exposing HepG2 cells and Caco-2 cells to oxygen radicals after 0.5, 1, 2, 4, 8 and 24 hours. Pathways were defined for genes present in these temporal expression curves and can be found in Table 4.

Table 4 (A) Significantly regulated pathways based on (A) overlapping genes, (B) unique genes in HepG2 cells and (C) unique genes in Caco-2 cells. A summary of significantly ($p<0.05$) regulated pathways as indicated by MetaCore is shown.

A. Overlapping pathways	
Pathways	**Cluster***
Chromosome condensation in prometaphasis	1
Nucleotide excision repair	1
WNT signaling pathway. Part 2	1
IFN gamma signaling pathway	1
Butanoate, Lysine, Propionate and Tryptophan metabolism	2
IL-13 signaling via PI3K-ERK	3
MIF-induced cell adhesion, migration and angiogenesis	4
TGF-beta-dependent induction of EMT via MAPK	5
IL-17 signaling pathways	5
B. Unique pathways in HepG2 cells	
Pathways	**Cluster**
Transition and termination of DNA replication	1
Start of DNA replication in early S phase	1
Mismatch repair	1
AKT signaling	1
Bile acids regulation of glucose and lipid metabolism via FXR	2
Role of IL-8 in angiogenesis	2
NOTCH1-mediated pathway for NF-KB activity modulation	2
P53 signaling	2
Activation of ERK by Kappa-type opioid receptor	3
Ceramides signaling pathway	3
IL-1, CD28, CD16 and IL-2 signaling	4
TGF-beta receptor signaling	4
Brca1 as a transcription regulator in DNA damage	5
C. Unique pathways in Caco-2 cells	
Pathways	**Cluster**
Cytoskeleton remodeling	1
IL-2 activation and signaling pathway	1
Role of IL-8 in angiogenesis	1
BAD phosphorylation	1
CCR4-induced chemotaxis of immune cells	2
IL-1, IL-15, IL-12 and IL-17 signaling	3
AKT signaling	3
Start of DNA replication in early S phase	4
NGF activation of NF-kB	5
IL-1, IL-18, IL-22, IL-12, IL-10 and IL-15 signaling	5
P53 signaling	5

** Defined cluster as presented in Figure 4*

Discussion

In this study, we examined the whole-genome gene expression of HepG2 and Caco-2 cells after exposure to reactive oxygen species at six different time points (0.5, 1, 2, 4, 8 and 24h). Correspondences and differences between HepG2 and Caco-2 cells were evaluated to improve our knowledge of cell type specific effects of oxidative stress and its effects on transcriptomics.

Although different array platforms were used, it has been described that high levels of concordance and reproducibility between different platforms [27, 28] exist when handling the data in a similar manner. In this way, we have to opportunity to obtain and compare cell type-specific gene expression changes in HepG2 and Caco-2 cells. Data from menadione and H_2O_2 exposure in HepG2 and Caco-2 cells were combined to evaluate global oxidative stress responses on a molecular level. Approximately, 3100 differences and 1800 overlaps in gene expression were evident between HepG2 and Caco-2 cells upon challenge by oxidative stressors. Despite the higher number of differently expressed than overlapping genes, most oxidative stress-related genes were differentially expressed in both cell types, moreover, expression of CAT, HMOX1 and GCLC was similar over time in HepG2 compared to Caco-2 cells. Furthermore, transcription factors NRF2 and NF-κB, both involved in first responses to oxidative stress, were differentially expressed in both HepG2 and Caco-2 cells. NRF2 can be activated directly by oxidative stress [30] or by map kinases such as ERK, JNK, PKC and PI3K/AKT which also activate NRF2 by inhibiting binding of KEAP1 [31]. Pathway analysis shows that during oxidative stress, PI3K/AKT signaling is activated in Caco-2 cells and PKC signaling is upregulated in HepG2 cells, so this may indicate a direct and indirect activation of NRF2 respectively. NF-κB, on the other hand, was negatively regulated by oxidative stress at 24h of exposure, however, different expression levels over time were observed between HepG2 and Caco-2 cells. Oxidative stress-regulated activation of NF-κB can be cell type-specific and might differ over time [32]. In addition, oxidative stress can inhibit basal NF-κB expression, possibly by oxidation of IKK or downregulation of AKT [33, 34], however these mechanisms cannot be confirmed by our transcriptomics data. A potential cross-talk between oxidative stress regulated NF-κB and NRF2 activation as have been investigated can be an alternative mechanism (Figure 2), suggesting HMOX1 upregulation by NRF2 as a key player in inhibiting the inflammatory effects of NF-κB [35]. On the contrary, it is believed that NF-κB could directly inhibit NRF2 activity at the transcription level [36]. Our results show that a higher expression of NRF2 is associated with a lower NF-κB expression, supporting the hypothesis of an anti-inflammatory effect of NRF2.

An important difference is that expression levels and/or direction of overlapping genes were mostly not similar in the two cell types. For example, the 0.5 and 1h time point in Caco-2 cells clustered with the 2 and 4h time point of

HepG2 cells, indicating a slower metabolic rate in this liver carcinoma cell line. However, DTW showed genes, such as GSTA4, with a delayed expression profile in Caco-2 cells, indicating differences in transcriptional regulation and not in metabolic rate between these cell lines. In addition, expression of AHR and MYC in Caco-2 cells was downregulated in response to oxidative stress, while, at the same time, they were upregulated in HepG2 cells following oxidant exposure. In a study of Liu, Wu [37], results indicate that AHR upregulation is associated with hepatic tumor invasion, where on the contrary, a recent study of Furumatsu, Nishiumi [38] shows that AHR activation has a protective role in IBD, and downregulation can aggravate colitis, which is one of the main risk factors in the development of colon cancer [39]. Changes in MYC expression on the other hand can have very different cellular outcomes, since it regulates 15% of all genes [40]. These differences in expression direction and affected genes between HepG2 and Caco-2 cells indicate a cell-specific response to oxidative stress, which may lead to very diverse downstream effects. This may be an explanation for the high number of unique affected genes between HepG2 and Caco-2 cells. In addition, these cell-specific responses can also be translated to differences in anti-oxidant mechanisms, DNA damage responses, inflammatory responses and cell cycle changes. This was confirmed by the observation that cell cycle changes in HepG2 cells were different compared to Caco-2 cells and correlated with apoptotic pathways whereas cell cycle changes in Caco-2 cells correlated with immune responses and metabolism. This indicates that oxidative stress induced cellular damage differs between cell types and can lead to a very different cell fate.

Next to these cell-specific responses following oxidative stress, different pathways are found to be similarly affected in both HepG2 and Caco-2 cells. Interestingly, the WNT signaling pathway is significantly changed in both cell lines and involved genes do also follow the same expression profile over time. WNT signaling has been described as being a key player in the development of colon cancer [41], but is also involved in hepatocellular carcinoma [42]. Also cellular processes such as cell cycle- and DNA damage responses, including chromosome condensation and NER, were similarly downregulated over time in HepG2 and Caco-2 cells. Pathways such as calcium signaling, HP1 induced transcriptional silencing and EMT induction by TGFβ, on the other hand, seems to be HepG2 cell-specific in response to oxidative stress. Protein folding, oxidative phosphorylation and different pathways of EMT were more Caco-2 cell-specific responses to oxidative stress. EMT is an important step in tumor progression and has been linked to chronic inflammation and oxidative stress [43, 44], however, cell type specific induction of EMT after oxidative stress has not been investigated yet. In addition to these findings, results showed significant pathways containing unique and overlapping DEGs, indicating that similar pathways were affected in both cell types, however, by affecting different genes in a particular pathway.

In conclusion, by comparing transcriptomics data of HepG2 and Caco-2 cells exposed to oxidants, more differences than common genes and pathways were identified, possibly due to differences in transcriptional regulation or cell type specific genotype. First response pathways to oxidative stress were similar in both cell types, however, the presence of cell specific pathways, which are of pathophysiological importance, indicates that oxidative stress induces different effects and outcomes in different cell types. Furthermore, time related events after oxidative stress differ between HepG2 and Caco-2 cells, indicating a cell type-specific regulatory response to oxidants, leading to different downstream effects and cascade signaling. Combining this with differences in transcriptional expression over time, this work can contribute to obtain a better molecular understanding of tissue-specific toxicity and cancer.

Supplementary data

You can download all supplementary data using following URL:
https://mega.co.nz/#F!SocUBQzD!-d7na22SyBLbV-zwPJs8VQ

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CHAPTER 4

Oxidative stress mechanisms do not discriminate between genotoxic and non-genotoxic carcinogenesis

Submitted

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Abstract

Chemical carcinogenesis is mainly linked with genotoxic actions such as DNA mutations, but may be complemented by non-genotoxic events; here oxidative stress has been suggested to play a key role. However, this may be different between particular modes-of-action, *e.g.*, genotoxic (GTX) versus non-genotoxic (NGTX) carcinogenesis. To evaluate this, we examined oxidative stress-related changes in gene expression, cell cycle distribution and (oxidative-) DNA damage induced by GTX-, NGTX- and non-carcinogens in human hepatoma cells (HepG2) at multiple time points (4-8-24-48-72h). Two GTX (Azathriopine, AZA/Furan) and NGTX (Tetradecanoyl-phorbol-acetate, TPA/Tetrachloroethylene, TCE) carcinogens as well as two non-carcinogens (Diazinon, DZN/D-mannitol, Dman) were selected, per class one compound was deemed to induce oxidative stress and the other not.

TPA appeared to cause oxygen radical formation, cell cycle changes and oxidative DNA damage as also did AZA and DZN; however, these compounds did also induce double DNA strand breaks while TPA did not. In addition, AZA, TPA and DZN induced a 10-fold higher number of gene expression changes over time compared to Furan, TCE or Dman treatment. A major fraction of these genes appeared commonly expressed between AZA, TPA and DZN which were specifically involved in oxidative stress, DNA damage and immune responses. On the contrary, differences in gene expression between these GTX and NGTX carcinogens did not correlate to oxidative stress or DNA damage, but could instead be assigned to compound-specific characteristics. Therefore, oxidative stress may represent the underlying cause for increased risk of liver toxicity and even carcinogenesis, however, it does not discriminate between GTX and NGTX carcinogens.

Introduction

Hepatic carcinogenesis is associated with a range of risk factors but underlying mechanisms-of-action tend to be very complicated and till date, remain not fully understood. Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer worldwide and evolves from different genetic lesions at regulatory sites [1]. However, causal factors of hepatocellular carcinoma mainly origin from the environment rather than from specific gene mutations genetically inherited [2]. Hepatitis B and C are still considered the major risk factors for HCC (70-80%) and have thus been extensively studied [3, 4], but also exposure of the liver to chemicals and drugs may induce or promote HCC [5]. Based on the mode of action by which these compounds induce hepatocarcinogenesis, they are generally classified as either genotoxic (GTX) or non-genotoxic (NGTX) carcinogens [6]. However, insights into mechanisms involved in NGTX carcinogenesis are considerably less advanced than for GTX carcinogens [7].

One molecular factor considered important in liver carcinogenesis is the formation of reactive oxygen species (ROS) [5, 8]. Compounds may induce oxidative stress by forming reactive oxygen species (ROS) directly, by increasing endogenous cellular generation of ROS, for instance by upregulating enzymatic metabolism, or by decreasing antioxidant and DNA repair mechanisms. Where there is consensus that GTX carcinogens act by directly damaging DNA, oxidative stress being of less or even no importance, results from rat studies suggest that endogenously induced oxidative stress plays a key role in the mode of action of NGTX carcinogens [7, 9, 10]. Enzyme systems such as the NADPH-oxidase complex, cyclooxygenase, xanthine-oxidase, lipoxygenase, cytochrome-P450 and peroxisomes, as well as inflammatory cells are sources of endogenous ROS [5, 11] which may be affected by NGTX carcinogens. Upon exposure to NGTX carcinogens, intracellular accumulation of ROS from endogenous and exogenous sources that are not handled by antioxidants such as superoxide dismutase, catalase, or other phase II genes activated by nuclear factor (erythroid-derived 2)-like 2 (NRF2), may result in oxidative stress. This subsequently leads to damage of different cell structures resulting in DNA mutations, chromosome instability and eventually cancer initiation and promotion [12].

In a previous global transcriptomic study using a human hepatoma cell line (HepG2 cells), we identified a signature of seventeen upregulated genes after exposure to different types of oxygen radicals [13]. As most of these genes are involved in hepatocarcinogenesis and/or oxidative stress, it is of interest to investigate the relation between upregulation of these genes and oxidative stress induced by NGTX carcinogens in comparison to GTX carcinogens. Consequently, for the present study, compounds were selected with regard to their ability to induce oxidative stress based on data on the expression of these genes obtained from a whole genome gene expression database generated from HepG2 cells

exposed to 62 GTX and NGTX compounds [14]. HepG2 cells were used as an *in vitro* model for the liver, the most important target for reactive metabolites from chemical carcinogens. Their ability to metabolize xenobiotics [15] although at a lower metabolic capacity compared to primary hepatocytes [16], absence of p53 mutations [17], and their easy usability and reproducibility in comparison to, for example, primary human hepatocytes, make these cells a convenient alternative for *in vivo* testing of *e.g.*, GTX and NGTX compounds [14]. For this study, two GTX carcinogens, two NGTX carcinogens and two non-carcinogens were selected, within each class one compound inducing upregulation of genes from this oxidative stress-related gene set and the other not. Since probably differences in the kinetics of ROS formation exist, temporal cellular changes induced by GTX and NGTX carcinogens and non-carcinogens were analyzed at five different exposure times, 4h, 8h, 24h, 48h and 72h in HepG2 cells. ROS generating capacities of these compounds were measured using electron spin resonance (ESR-) spectroscopy. Significantly induced molecular pathways were further correlated with functional effects *e.g.*, ROS levels, (oxidative-) DNA damage and cell cycle progression.

Material and methods

Compound selection

Two GTX-, NGTX- and non-carcinogens (as classified *in vitro* and *in vivo* using different criteria as described before [14]), were selected based on their gene expression profile in response to oxidative stress. This selection was based on a previously published gene-set of 17 genes (BIK, AKR1C2, GCLC, GCLM, GSR, LIF, RAP1GAP, SQSTM1, GCNT3, RRAS2, SLC7A11, ASF1A, ASKR1B10, FBXO30, AGPAT9, SRXN1, PTGR1) related to oxidative stress in HepG2 cells [13]. Based on previously obtained transcriptomics data of HepG2 cells treated for 24h and 48h [14, 18], log2 ratios of these 17 genes were selected. A gene was classified as a differentially expressed gene (DEG) if the absolute average fold change of a gene was [>1.2] and the p-value <0.05. The total number of upregulated DEGs was scored and percentages were calculated for each individual compound compared to the total number of selected genes (Supplementary Data 1). For each carcinogen class an oxidative gene set-inducing compound (highest %) and a non-oxidative gene set-inducing compound (0%) was selected (Supplementary Data 2). This selection resulted in six compounds; 1. Azathioprine (AZA, 88%, oxidative stress inducer) and Furan (0%) as GTX carcinogens, 2. Tetradecanoyl phorbol acetate (TPA, 44%, oxidative stress inducer) and Tetrachloroethylene (TCE, 0%) as NGTX carcinogens, and 3. Diazinon (DZN, 63%, oxidative stress inducer) and D-mannitol (Dman, 0%) as non-carcinogens.

Cell culture and treatment

HepG2 cells (ATCC, LGC logistics), were cultured in six-well plates and 35*10 mm dishes in the presence of minimal essential medium (MEM) plus GlutaMAX supplemented with 1% non-essential amino acids (NEAA), 1% sodium-pyruvate, 1% penicillin/streptomycin, and 10% fetal calf serum (FCS) (all from Gibco BRL, Breda, The Netherlands). The cells were incubated at 37°C with 5% CO_2. When obtaining 80% confluence, the medium was replaced by medium containing the IC20 concentration [14] of AZA (250µM, Sigma-Aldrich), Furan (2mM, Sigma-Aldrich), TPA (500nM, Sigma-Aldrich), TCE (2mM, Sigma-Aldrich), DZN (250µM, Sigma-Aldrich) and Dman (250µM, Sigma-Aldrich) or with the corresponding control treatment (DMSO, EtOH, or HBSS 0.5%). Time-matched control cells were treated in an identical manner without addition of a compound.

Identification and levels of radical formation

Radical formation in HepG2 cells was measured after 0.5, 2, 4, 8, 24, 48 and 72h exposure to AZA, Furan, TPA, TCE, DZN and Dman using electron spin resonance (ESR) spectroscopy in combination with the spin probing technique using 250µM 1-Hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine (CPH, Noxygen, Elzach, Germany) which forms CP radicals in the presence of ROS. CPH was added to control and exposed cells 0.5h prior to measurements where the triple-line spectrum of the CP radical was detected by ESR spectroscopy [19]. Instrument settings were 50mW of microwave power, 1G of amplitude modification, 100 kHz of modulation frequency and 60G sweep widths. Spectra were quantified (in arbitrary units) through peak surface measurements of the triple peak spectra of CPH (AUC = Area Under the Curve) using the WIN-EPR spectrum manipulation program [19].

Cell cycle distribution, double-strand breaks and oxidative DNA damage

Methanol-fixated cells were labeled for double strand breaks using the γH2AX staining as previously been described [20]. As positive and negative controls, cells were incubated with 5µM Etoposide (Sigma-Aldrich) and the corresponding solvent controls, respectively [21].

For quantifying 8-OHd, the OxyFLOW DNA-damage kit (Calbiochem, USA) was applied as described in the manufacturer protocol. Methanol-fixated cells were incubated for 1h with the FITC-labelled anti-8-OHdG antibody diluted 1:30 in wash solution. As negative controls, cells were incubated with the corresponding solvent controls. Cell cycle distribution was measured simultaneously using propidium iodide (PI) staining as previously described [22].

Cell cycle, γH2AX signals and 8-OHdG signals were analyzed using ModFit LT for Mac (version 2.0). Cells in the G1, S or G2/M phase were expressed as a percentage of the total number of cells (10000 cells/sample). Cells with significant levels of γH2AX and 8-OHdG positive signals were presented as a percentage of total cells.

Total RNA isolation and microarray experiments

Total RNA was extracted from HepG2 cells using 0.5mL QIAZOL according to the manufacturer's instructions. RNA quality was assessed by automated gel electrophoresis on an Agilent 2100 Bioanalyzer (Agilent Technologies, Netherlands).

cDNA was prepared using Affymetrix synthesis and labelling kits as described before (Affymetrix, Santa Clara) [15]. cRNA targets were hybridized on high-density oligonucleotide gene-titan chips (Affymetrix Human Genome U133 Plus PM GeneTitan 24 arrays) according to the Affymetrix Eukaryotic Target Hybridization manual. The Gene-Titan arrays were hybridized washed and stained using the Gene-Titan hybridization, wash- and stain kit for 3' IVT Arrays and Gene-Titan Operating Software and scanned by means of an Affymetrix Gene-Titan scanner. Normalization quality controls, including scaling factors, average intensities, present calls, background intensities, noise, and raw Q values, were within acceptable limits for all chips.

Whole genome gene expression analysis

Re-annotation and normalization

Data from a total of 135 arrays were obtained, and Robust Multi-array Average (RMA) normalized and re-annotated to custom CDF (version 14.1.0) files using the arrayanalysis tool (http://arrayanalysis.org). In addition, after correcting for platform probes, 17143 genes were analyzed for the number of absence calls in the three replicates per treatment. Genes that contained two or more absence calls within the three replicates for all the treatments as well as in controls were omitted from the data. The data discussed in this publication have been deposited in NCBI's gene expression omnibus (Edgar et al., 2002) and are accessible through GEO series accession number GSE58235:
http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE58235

Data filtering

The intensities of the filtered data sets were log2 transformed, and subsequently, log ratios of treated versus controls were calculated. DEGs for each experimental group were selected using the following criteria: (1) log ratio of −0.26 or >0.26 (i.e., absolute fold change of 1.2) for the average of the three replicates within the experimental group, (2) same direction of the log ratio for all replicates, (3) intensity of log2 values >6 for at least 2 out of 3 replicates, and (4) a p value of <0.05 determined using the Student's t-test. No FDR was used since it has been reported that reproducibility of microarray data is higher if criteria such as fold change are applied [23].

Time series analyses by STEM

For identification of genes co-regulated time-dependently and clustering with markers for oxidative stress, the software tool “Short Time-series Expression Miner” (STEM 1.3.8; http://www.cs.cmu.edu/~jernst/stem/) [24] was used. Criteria used were described before [25]. For correlation analysis, data from γH2AX, 8-oxodG and cell cycle distribution levels were transformed into log2 ratios.

Pathway analysis of DEGs

MetaCore (GeneGo, San Diego, CA) was used to identify and visualize the involvement of the DEGs and transcription factors in the biological processes that may be affected at the level of pathways ($p < 0.05$).

Statistical analysis

Data are presented as means ± SD. Statistical analyses of changes, for each time point compared to control, were performed using a unpaired two-tailed student’s t-test (in levels of radical formation) or a paired two-tailed student’s t-test (in (oxidative-) DNA-damage and cell cycle distributions) with statistical significance set at $p < 0.05$.

Results

Detection of ROS formation and (oxidative-) DNA damage

ROS formation after chemical exposure

In the presence of HepG2 cells and the spin probe CPH, AZA exposure for 24, 48 and 72h, as well as DZN exposure at 48 and 72h, resulted in significantly increased levels of cellular ROS production (Figure 1). Surprisingly, TPA did not induce a significant increase of ROS levels. As expected, the non-oxidative stress inducing compounds TCE, Furan and Dman did not cause ROS formation. Based on these results, following incubation periods were selected for further studies on (oxidative-) DNA damage and cell cycle distribution: 8, 24, 48 and 72h. In addition, 4h of exposure was included within the whole genome gene experiments.

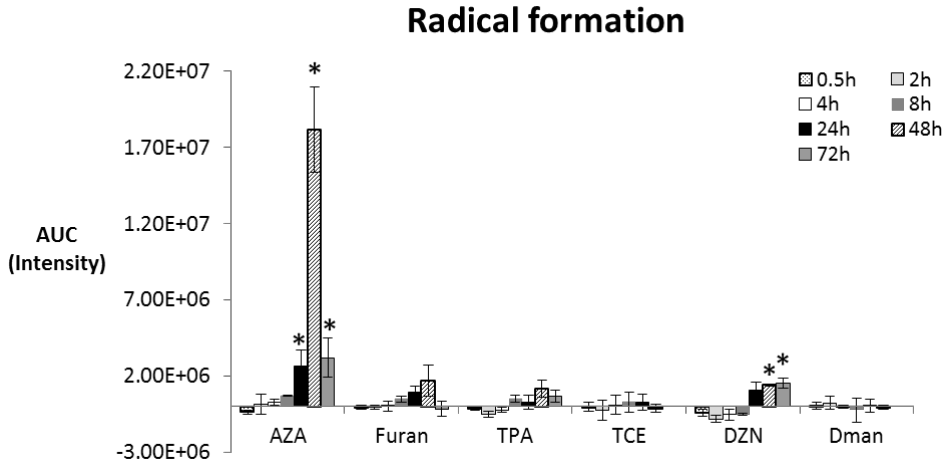

Figure 1 Levels of time dependent radical formation after exposure to AZA, Furan, TPA, TCE, DZN and Dman using ESR in combination with the spin probe CPH. Results are corrected for background levels in control conditions (n=3, $p<0.05$). AUC: area under the curve of radical-specific signals; time (h)

(Oxidative-) DNA damage after chemical exposure

AZA, as well as the non-carcinogen, DZN, induced a significant increase in 8-OHdG and γH2AX levels after 24, 48 and 72h (Figure 2A and B). 24 and 48h TPA treatment resulted in significantly elevated levels of 8-OHdG but not of ROS levels (Figure 1 and 2A). However, none of these compounds induced (oxidative-) DNA damage after 8h exposure (data not shown). Exposure to Furan, TCE or Dman did not result in a significant increase in (oxidative-) DNA damage over time (Figure 2). Overall, our findings show that these selected GTX-, NGTX- and non-carcinogens except for NGTX carcinogen TPA, are able to induce oxidative DNA damage as well as double DNA strand breaks.

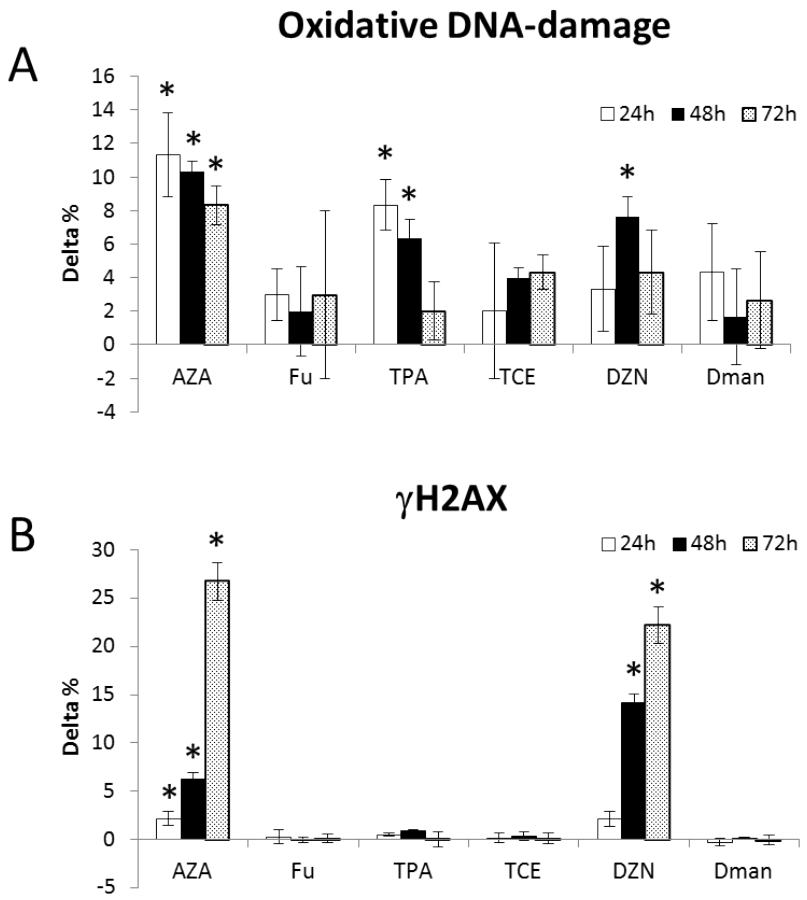

Figure 2 DNA damage induced by AZA, Furan, TPA, TCE, DZN and Dman using flow cytometry. (A) Levels of 8-OHdG after 24, 48 and 72h exposure. (B) Levels of γH2AX after 24, 48 and 72h exposure. Values were corrected for blank levels. (n=3, $p<0.05$)

Cell cycle distribution after chemical exposure

With regards to cell cycle changes, 48h (data not shown) and 72h (Figure 3) of AZA exposure and 24h of Furan treatment (data not shown) resulted in a significant decrease in G1 phase, indicating G1/S phase arrest. TPA exposure did also affect the cell cycle by inducing G1 phase arrest after 24, 48 and 72h of exposure, whereas TCE treatment had no effect on cell cycle distribution. DZN exposure affected the cell cycle by simultaneously decreasing the G1 phase and increasing the S phase at 24h (data not shown) and decreasing the G2 phase at 72h (Figure 3) while Dman treatment induced an increase in S phase after 48h exposure, indicating that both DZN and Dman are capable of inducing S phase arrest in HepG2 cells. These results show that S phase arrest by these GTX- and non-carcinogens can also develop without concomitant oxidative stress, whereas G1 phase arrest was specifically induced by the NGTX carcinogen TPA.

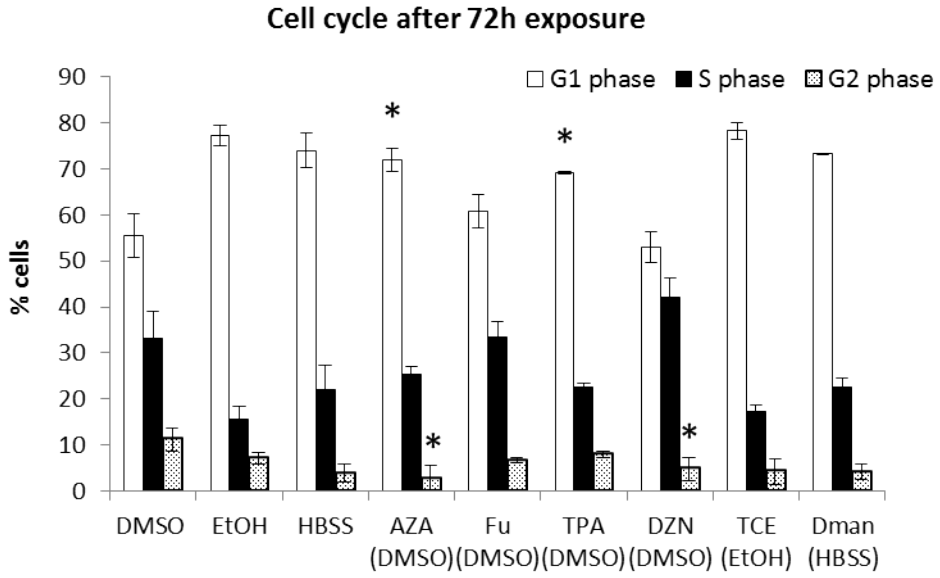

Figure 3 Levels of different cell cycle phases: G1 phase, S phase and G2/M phase distribution after 72h AZA, Furan, TPA, TCE, DZN and Dman exposure in HepG2 cells using flow cytometry. (n=3, $p<0.05$)

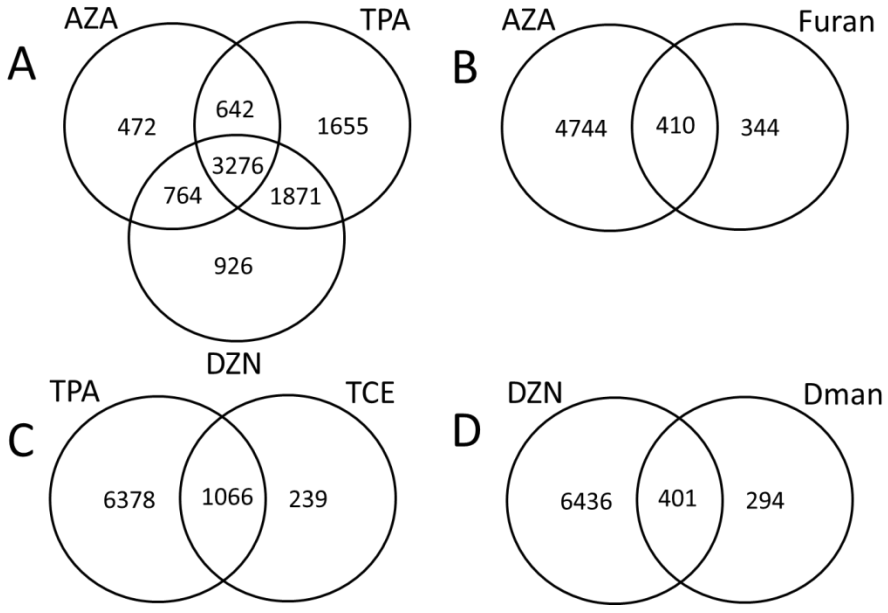

Figure 4 Venn diagrams of differentially expressed genes after exposure to (A) AZA, TPA and DZN, (B) AZA and Furan, (C) TPA and TCE and (D) DZN and Dman over all time points showed unique-and overlapping genes.

Table 1 Selection of most significantly regulated processes and involved pathways based on 3276 common DEGs induced by AZA, TPA and DZN treatment. A summary of significantly ($p<0.05$, FDR<5%) regulated pathways and related cellular processes as indicated by MetaCore is shown.

Pathways and cellular processes	p value
Cell cycle and its regulation	
The metaphase checkpoint	5.661E-12
Start of DNA replication in early S phase	3.307E-10
Chromosome condensation in prometaphase	8.742E-09
DNA-damage response	
ATM/ATR regulation of G1/S checkpoint	2.242E-07
Role of Brca1 and Brca2 in DNA repair	5.396E-07
ATM/ATR regulation of G2/M checkpoint	2.141E-05
Apoptosis	
Endoplasmic reticulum stress response pathway	4.333E-07
Granzyme A signaling	3.710E-06
HTR1A signaling	5.690E-05
Tissue remodeling and wound repair	
TGF, WNT and cytoskeletal remodeling	4.695E-12
MIF-induced cell adhesion, migration and angiogenesis	2.226E-08
Chemokines and adhesion	4.851E-08
Metabolic diseases	
Role of ER stress in obesity and type 2 diabetes	2.956E-07
Role of adipose tissue hypoxia in obesity and type 2 diabetes	1.196E-04
Immune system response	
IFN gamma signaling pathway	4.264E-09
Oncostatin M signaling via MAPK in human cells	9.139E-09
C5a signaling	6.808E-07
Oxidative stress regulation	
MIF-induced cell adhesion, migration and angiogenesis	2.226E-08
IL-18 signaling	4.633E-06
Glucocorticoid receptor signaling	2.990E-04
Protein degradation	
Role of Parkin in the Ubiquitin-Proteasomal Pathway	1.479E-03
Putative SUMO-1 pathway	1.172E-03
Transcription regulation	
CREB pathway	2.033E-07
Role of AP-1 in regulation of cellular metabolism	9.489E-05
Receptor-mediated HIF regulation	1.314E-04
Angiogenesis	
Role of IL-8 in angiogenesis	6.978E-08
FGFR signaling pathway	1.280E-07
PDGF signaling via STATs and NF-kB	1.547E-06

Oxidative stress-related gene expression changes

Overall, oxidative stress inducers, AZA (5155 DEGs), TPA (7445 DEGs) and DZN (6838 DEGs), induced a 5-10 fold higher number of DEGs compared to Furan (755 DEGs), TCE (1306 DEGs) and Dman (696 DEGs) (Figure 4).

DEGs after exposure to oxidants vs non-oxidants

When comparing gene expression after AZA, TPA and DZN treatment, 3276 genes were found to be commonly modified (Figure 4A) and these could be assigned to 277 significant pathways in particular involved in cell cycle changes, apoptosis, DNA damage and immune responses (Table 1).

These common DEGs include among others (in)direct oxidative stress-related genes such as NRF2, KEAP1, NF-κB, GCLC, GCLM, GSTA4, NQO1, HMOX1, MSH6, CDKN1A and CCND1. Unique genes on the other hand, referred to more compound-specific responses (Table 2) such as 25 pathways mainly involved in the cytoskeleton and developmental processes induced by AZA (Table 2A). 55 pathways induced by TPA on the other hand, had a stronger effect on signal transduction, apoptosis/survival responses and developmental processes (Table 2B), while 16 pathways following DZN treatment were mainly involved in immune responses (Table 2C).

Overall, the high number of DEGs commonly induced by AZA, TPA and DZN, appeared to be mainly involved in oxidative stress-related events, thus indicating an important role of oxidative stress responses.

DEGs in GTX vs NGTX carcinogen treatments

By comparing GTX carcinogens, Furan and AZA, 410 DEGs were found to be affected by both compounds (Figure 4B) which are mainly involved in immune and developmental processes (Figure 5A). Common DEGs included oxidative stress-related genes such as GCLC, GCLM, HMOX1 and GSR. However, where those genes were upregulated upon AZA challenge, they were downregulated after Furan treatment.

Table 2 Selection of most significantly regulated processes and involved pathways based on unique DEGs induced by (A) AZA, (B) TPA and (C) DZN treatment. A summary of significantly ($p<0.05$, FDR<5%) regulated pathways and related cellular processes as indicated by MetaCore is shown.

Pathways and cellular processes	p value
A. Significant affected pathways after AZA treatment	
Cytoskeleton remodeling	
Cytoskeleton remodeling	9.035E-06
TGF. WNT and cytoskeletal remodeling	1.800E-05
FAK signaling	1.002E-03
Role of Activin A in cytoskeleton remodeling	2.337E-03
Development	
TGF-beta-dependent induction of EMT via MAPK	3.719E-05
Thromboxane A2 pathway signaling	4.737E-05
CNTF receptor signaling	8.529E-05
EGFR signaling via small GTPases	9.678E-04
B. Significant affected pathways after TPA treatment	
Signal transduction	
Activation of PKC via G-Protein coupled receptor	3.526E-05
Angiotensin signaling via STATs	1.201E-04
JNK pathway	1.639E-04
Regulation of p38 and JNK signaling mediated by G-proteins	5.187E-04
Apoptosis and survival	
Anti-apoptotic TNFs/NF-kB/Bcl-2 pathway	2.692E-05
BAD phosphorylation	1.639E-04
APRIL and BAFF signaling	2.619E-03
Ceramides signaling pathway	3.043E-03
Development	
A3 receptor signaling	5.560E-04
G-CSF signaling	5.560E-04
TGF-beta receptor signaling	6.491E-04
A2B receptor: action via G-protein alpha s	6.491E-04
C. Significant affected pathways after DZN treatment	
Immune system response	
Lectin induced complement pathway	2.418E-06
Function of MEF2 in T lymphocytes	2.886E-06
Classical complement pathway	4.061E-06
IFN gamma signaling pathway	3.257E-04

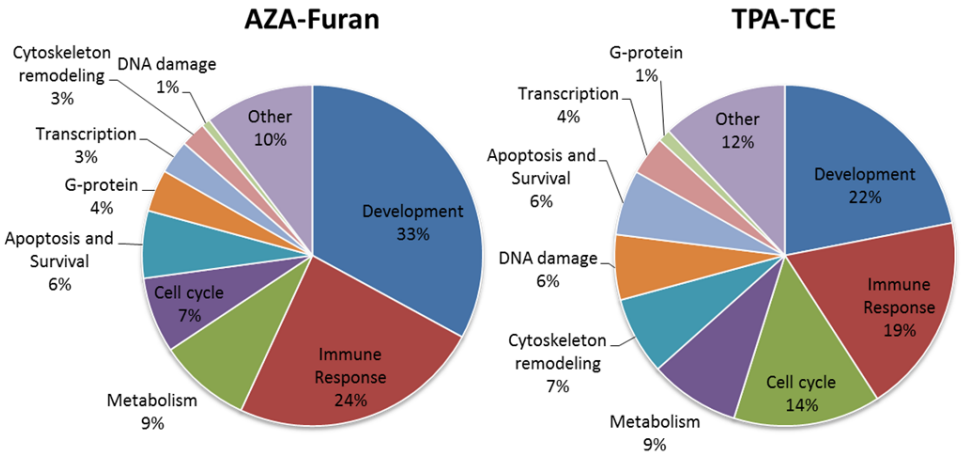

Figure 5 Pie chart representation of pathway analysis for DEGS of (A) AZA-Furan and (B) TPA-TCE exposure in HepG2 cells. Pie charts show the percentage of significantly influenced cellular components with a cut-off of P-value<0.05; FDR<0.5. A colour version of this figure is available online.

NGTX carcinogens TPA and TCE affected a total of 1066 common DEGs especially involved in developmental, immune and cell cycle processes (Figure 4C, Figure 5B). Common genes include NRF2, CAT and different cyclin dependent kinases (CDK), which were significantly downregulated while NF-κB and p21 were found to be upregulated.

Exposure to non-carcinogens, DZN and Dman, resulted in 401 common DEGs in HepG2 cells (Figure 4D) especially involved in signal transduction and developmental pathways (data not shown).

In conclusion, it appeared that both these GTX and NGTX-carcinogens are able to affect oxidative stress-related gene expression.

Time-related events induced by different oxidative stress inducing chemicals

Using STEM, each gene was assigned to a model profile that most closely matched with the temporal gene expression of that specific gene. DEGs resulting from AZA, TPA and DZN exposure were assigned to four, nine and six different significant time clusters respectively (data not shown), which may indicate that multiple gene expression-related processes were induced coherently over time by these chemicals.

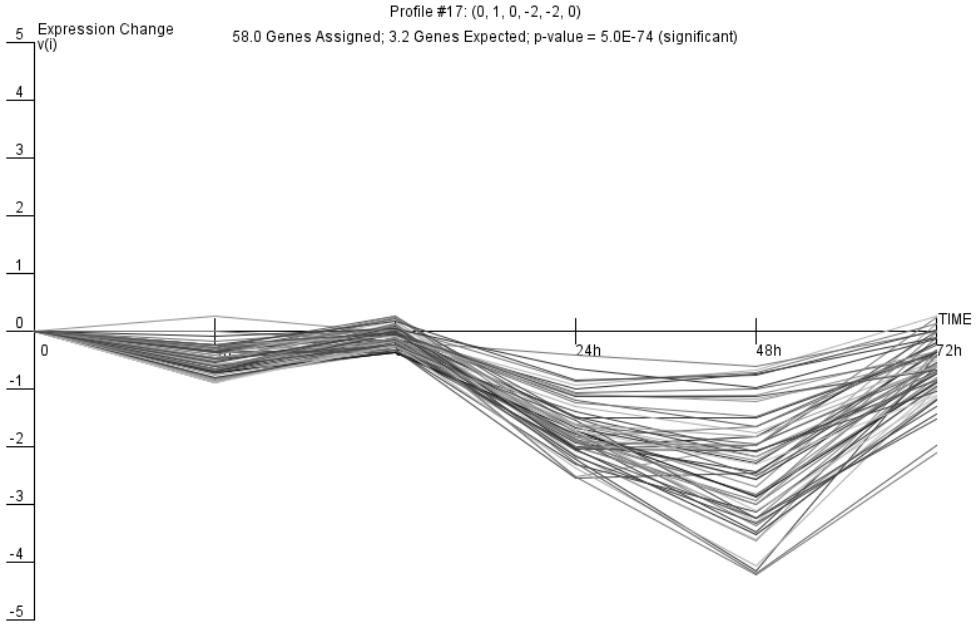

Figure 6 Correlating temporal gene expressions at 4, 8, 24, 48 and 72h of 58 common DEGs between AZA, TPA and DZN exposure in HepG2 cells generated by STEM.

Between these oxidative stress-inducing chemicals, one identical time cluster containing genes that were downregulated over time was identified (Figure 6). Moreover, 58 common DEGs in this time cluster were observed to have identical expression profiles after exposure to AZA, TPA and DZN (Supplementary data 3). Most of these genes appeared to be involved in cell cycle, DNA damage and repair processes.

In addition, DZN and AZA appeared to share 3 identical time clusters consisting of 1605 common DEGs, indicating a major resemblance of gene expression changes over time among these chemicals. Non-oxidative stress inducers (Furan, TCE and D-man) affected a significantly smaller amount of DEGs which were assigned to seven or more different time profiles which did not overlap with each other. This indicates that observed similarities between GTX and NGTX-induced pathway and gene regulation over time were mainly induced by oxidative stress.

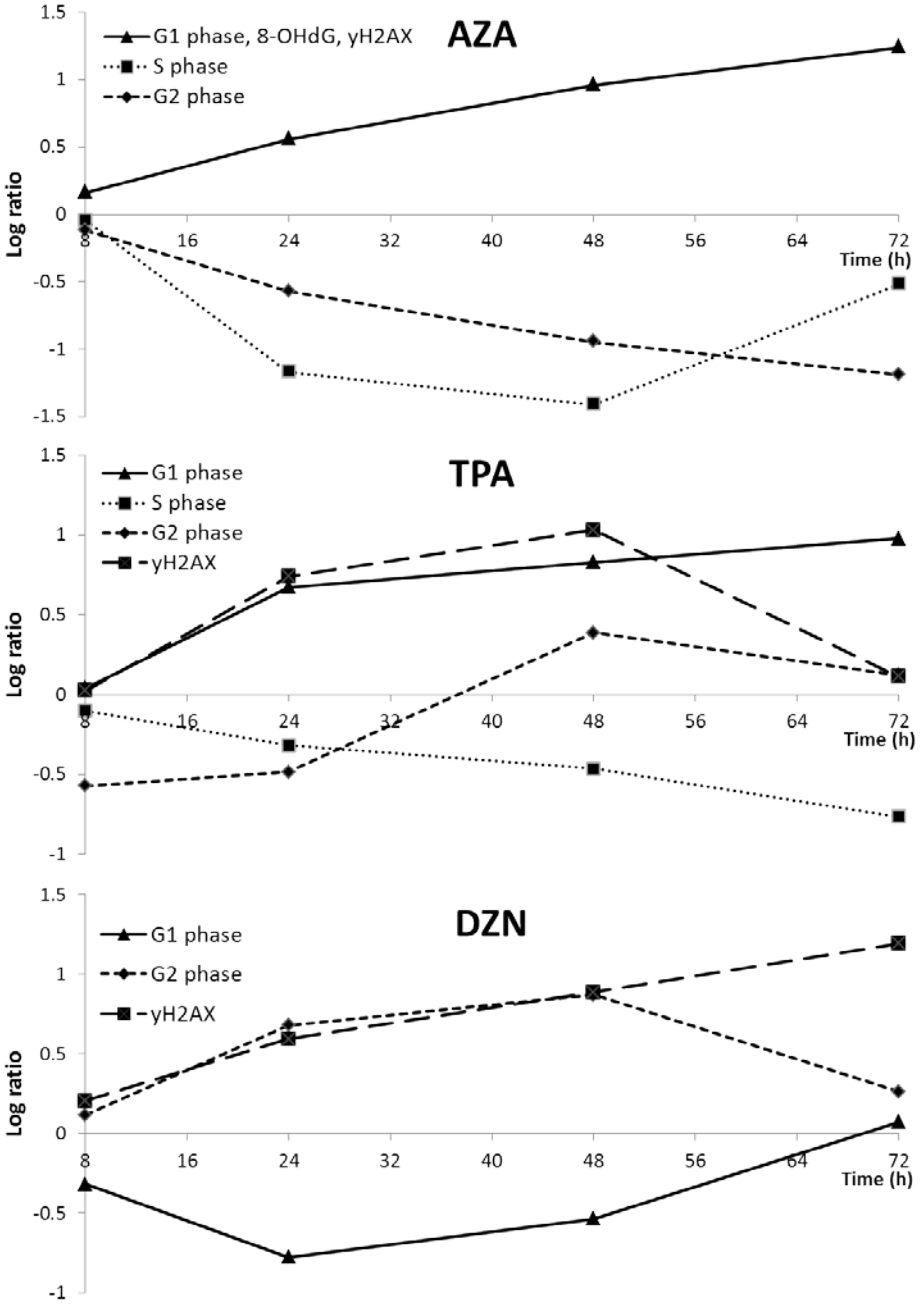

Figure 7 Average temporal gene expression correlating to levels of (oxidative-) DNA damage, and cell cycle distribution presented as time profiles generated by STEM after exposing HepG2 cells to (A) AZA, (B) TPA and (C) DZN during 8, 24, 48 and 72h.

Functional comparison of oxidative stress-related events

By using STEM, temporal gene expression changes were correlated to functional endpoints such as cell cycle and (oxidative-) DNA damage levels over time (Figure 7).

A high number of DEGs induced by AZA correlated with all phenotypic endpoints, comprising increased G1 phase levels, as well as 8-OHdG- and γH2AX levels (Figure 7A). MetaCore analysis of correlating DEGs returned several pathways involved in G1/S phase transition, apoptosis, but also pathways involved in cytoskeleton-, immune- and developmental processes were observed (Table 3A). In addition, decreasing S and G2 phase levels induced by AZA over time, correlated with genes involved in cell cycle- and DNA damage-related processes (Table 3A, Figure 7A). Cellular responses induced by AZA were especially affected at 24, 48 and 72h exposure, which can be associated with significant increased ROS levels induced by AZA at these time points. On the contrary, functional effects induced by non-oxidative GTX carcinogen Furan did not correlate significantly to DEGs.

Where TPA-induced oxidative DNA damage did not have any correlation with DEGs, increasing levels of G1 and G2 phases correlated to apoptotic processes and γH2AX levels correlated to immune responses, cytoskeleton and DNA damage responses such as BRCA1 regulated DNA repair (Figure 7B, Table 3B). Functional responses caused by the non-oxidative NGTX carcinogen TCE did not correlate significantly to any DEG.

Increasing G1 phase- and γH2AX levels induced by the oxidative non-carcinogen DZN over time, correlated to DEGs (Figure 7C) involved in relevant cell cycle pathways and DNA damage responses, but also immune responses and apoptotic pathways (Table 3C). γH2AX levels induced by DZN did also correlate to γH2AX levels induced by AZA. DZN-induced G2 and S phase changes correlated to different genes, however, these genes did not return any significant pathways. In addition, these effects after especially 48 and 72h of DZN exposure did also associate with significantly increased ROS formation by this compound during these time points. On the contrary, S phase changes induced by Dman did correlate with DEGs, and resulted especially in developmental pathways and cell cycle related pathways (data not shown).

These observations indicate that oxidative stress-related events can be associated with the presence or absence of increased ROS formation and were very similar between AZA, TPA and DZN and do not discriminate between GTX and NGTX carcinogens.

Table 3 Genes that clustered in the same gene expression cluster profiles with measured phenotypic endpoints such as, cell cycle and (oxidative-) DNA damage using STEM after (A) AZA, (B) TPA and (C) DZN exposure. Amount (#) and most significant ($p<0.05$, FDR<5%) processes and pathways as indicated by MetaCore.

A. Phenotypical anchoring after AZA administration			
Endpoint	**#**	**Processes**	**Most significant pathways**
G1 phase – 8-OHdG – γH2AX	262	Cell cycle	Regulation of G1/S phase transition
		Apoptosis	PKR in stress-induced apoptosis
		DNA damage	Brca1 as a transcription regulator
		Immune response	Oncostatin Mn IL-2, PKR and IL-6 signaling
		Cytoskeleton	TGF, WNT and Cytoskeleton remodeling
		Development	TGF beta receptor signaling, EGFR signaling
S phase	21	Cell cycle	Metaphase checkpoint, DNA replication in early S phase, APC and ESR1 in cell cycle regulation, Chromosome condensation
G2 phase	12	Cell cycle	Chromosome condensation in prometaphase
		Protein folding	Angiotensin system
B. Phenotypical anchoring after TPA administration			
Endpoint	**#**	**Process**	**Most significant pathways**
G1 phase	7	Apoptosis	TNF alpha signaling
G2 phase	4	DNA damage	Brca1 and Brca2 regulation in DNA repair
γH2AX	35	DNA damage	Brca1 as a transcription regulator
		Cytoskeleton	TGF, WNT and Cytoskeleton remodeling
		Immune response	IL1, MIF and IL-12 signaling
C. Phenotypical anchoring after DZN administration			
Endpoint	**#**	**Process**	**Most significant pathways**
G1 phase	35	Cell cycle	Start of DNA replication in early S phase, APC in cell cycle regulation, ESR1 regulation in G1/S phase transition
		DNA damage	Nucleotide excision repair, Brca1 and Brca2 in DNA repair
G2 phase	7	Cytoskeleton	TGF, WNT and Cytoskeleton remodeling
γH2AX	317	DNA damage	Brca1 as a transcription regulator
		Apoptosis	PKR in stress-induced apoptosis, HTR1A signaling
		Immune response	Oncostatin M, IFN gamma signaling

Discussion

In this study, oxidative stress-related mechanisms were compared between selected GTX-, NGTX- and non-carcinogens. The presence of cellular oxidative stress induced by AZA, TPA and DZN was confirmed by ESR spectroscopy, analysis of 8-OHdG levels and gene expression data (Table 4). This

demonstrates the relevance of the previously described gene list to predict oxidative stress-inducing capacities of unknown compounds [13]. Functional correlation over time showed the activation of repair and cell cycle processes simultaneously with induced (oxidative-) DNA damage and cell cycle arrest by AZA, TPA and DZN. In addition, these temporal oxidative stress-related events between these compounds were very similar over time, showing similar kinetics. These results indicate that oxidative stress-related mechanisms are not limited to non-genotoxic carcinogens and therefore, do not discriminate between these GTX and NGTX carcinogenesis.

GTX carcinogens

GTX carcinogens, AZA and Furan, need metabolization to exert their GTX characteristics. AZA is metabolized by the rate-limiting enzyme thiopurine methyltransferase (TPMT) to 6-mercaptopurine [26] which was found to be upregulated in our study. Furthermore, observed oxidative-DNA damage and simultaneously, early G1 phase arrest indicate the activation of repair mechanisms over time as demonstrated by correlating pathways related to cell cycle processes, apoptosis and developmental processes. Interestingly, since no GTX-related events were observed among pathways uniquely affected by AZA, we believe that for this compound, oxidative stress is very likely the cause of GTX carcinogenesis.

Surprisingly, exposure of HepG2 cells to Furan did not show any DNA damage or increase in oxidative stress-regulated genes/pathways, however, the presence of an S phase arrest over time indicates a response to cellular damage. While this compound has been classified as a GTX carcinogen *in vitro*, inconsistent results of genotoxicity *in vivo* however exist [27, 28].

Table 4 Overview of effects induced by AZA, Furan, TPA, TCE, DZN and Dman

	Class	% ox. stress-related genes	ROS formation	8OHdG	γH2AX	Cell cycle	# genes
AZA	GTX carcinogen	88%	+	+	+	G1/S phase arrest	5155
Furan	GTX carcinogen	0%	-	-	-	S phase arrest	755
TPA	NGTX carcinogen	44%	-	+	-	G1 phase arrest	7445
TCE	NGTX carcinogen	0%	-	-	-	No effect	1306
DZN	Non-carcinogen	63%	+	+	+	S phase arrest	6838
Dman	Non-carcinogen	0%	-	-	-	S phase arrest	696

NGTX carcinogens

NGTX carcinogen TPA, a tumor promoter, exerts its toxic effects by activation of protein kinase C (PKC) and integrins which may lead to downstream ROS formation by NADPH oxidase activity [29]. Our results confirm this mechanism of action, since oxidative DNA damage was observed after 24h and 48h and at the same time PKC expression as well as its activation pathway appeared significantly upregulated. In addition, oxidative stress-related genes and pathways such as the NRF2 pathway, cell cycle processes and DNA damage responses correlated to functional endpoints.

TCE exposure did also induce changes in pathways involved in DNA damage and cell cycle while functional endpoints for oxidative stress effects were not affected. In a previous study, ROS formation and glutathione (GSH) depletion were detected after exposure to TCE in human lung cancer cells [30]. Possibly, GSH levels in HepG2 were not depleted completely, implying that ROS levels remained too low to induce functional endpoints. Gene expression of CYP genes however, appeared increased, so metabolic activation of TCE should have occurred [31]. In addition, NF-κB was upregulated after 8h exposure, while NRF2 and CAT were downregulated at that time, which was also observed after TPA exposure. Possibly NRF2 expression is directly downregulated by NF-κB as previously have been described [32]. Furthermore, NGTX carcinogens may also bind with high affinity to the aryl hydrocarbon receptor (AhR) (Beischlag et al. 2008; Mates et al. 2010), as demonstrated in gene expression data of TPA, where AHR expression was increased after all exposure periods. Also, over-expression of CYP genes, found to be upregulated after TCE treatment and known to be regulated by AHR, may also lead to the production of reactive oxygen species (Cederbaum et al. 2001; Mates et al. 2010).

The fact that mechanisms such as PKC activation and GSH depletion which lead to ROS formation, as well as response pathways such as cell cycle, immune, anti-oxidant and DNA damage responses, are shared by TPA and TCE, highlights that oxidative stress is an import key player in carcinogenic events induced by these NGTX compounds.

However, it is important to stress that temporal modifications of the NRF2 pathway, cell cycle processes and DNA damage responses were in common between TPA and AZA, as were oxidative DNA damage and cell cycle changes. This does not subscribe to the hypothesis that the induction of oxidative stress responses is a specific event in NGTX carcinogenesis.

Non-carcinogens

Non-carcinogen DZN is metabolized by oxidative desulfuration to diazoxon which can decrease CAT and SOD activity [33] which was also observed in our results. This may disturb the defense mechanism of the cell against free radicals dramatically, and as a result, NRF2, GCLC, GCLM and other first response mechanisms against oxidative stress were highly upregulated. Interestingly,

responses induced by non-carcinogen, DZN, were similar to those after exposure to GTX carcinogen, AZA. Both compounds induced significant ROS formation after 48 and 72h, however, ROS levels were much higher after AZA treatment. γH2AX levels on the other hand, correlated with each other as well as a large overlap of genes and pathways. Furthermore, both AZA and DZN exposure induced a large number of coherent processes that were similar over time and comprised DNA damage responses, cell cycle processes, immune responses, apoptotic/survival processes and cellular development. For these reasons, DZN appears to possess GTX characteristics which may point into the direction of a misclassification of this compound as has already been hypothesized in a previous study [34].

Results on DZN-induced oxidative stress responses suggest that oxidative stress is neither specific for GTX nor for NGTX carcinogens, and represents generic responses to compound metabolism.

In conclusion, all ROS forming compounds investigated in the present study, induced oxidative DNA damage, and shared a significant number of DEGs and temporal gene expression patterns especially involved in oxidative stress-related processes such as anti-oxidant functions, DNA damage, cell cycle changes, and immune responses as well as specific oxidative stress regulation processes. Differences in gene expression between these compounds on the other hand, did not directly correlate to oxidative stress, DNA damage or cell cycle processes, but could instead be assigned to compound-specific characteristics. This suggests that oxidative stress may not specifically represent a discriminative event between GTX and NTGX carcinogens. Moreover, cellular oxidative stress has a strong pronounced temporal effect on DNA damage, gene expression and cell cycle distribution, and for certain compounds, may represent the underlying cause for increased risk of liver toxicity and carcinogenesis.

Supplementary data

You can download all supplementary data using following URL:
https://mega.co.nz/#F!GglwnYqQ!ygVrRT-L03fnxS6GEYPZvQ

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CHAPTER 5

Dynamic interplay between the transcriptome and methylome in response to oxidative- and alkylating stress

Submitted

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Abstract

In recent years, it has been shown that free radicals do not only react directly with DNA, but also regulate epigenetic processes such as DNA methylation which may be relevant within the context of *e.g.*, tumorigenesis. However, how these free radicals impact on the epigenome, remains unclear. Therefore, we investigated whether methyl- and hydroxyl radicals, formed by *tert*-butyl hydroperoxide (TBH), are able to change DNA methylation patterns and how this interferes with the interplay with the transcriptome. We applied microarray-based technologies in combination with ESR spectroscopy and chemical identification of 5mC levels using LC-MS/MS, at multiple time points during TBH exposure of HepG2 cells. We found that methyl radicals are not able to function as methyl donors. Induced methylome changes rather represent an adaptive response to the oxidative stress-related reactions observed in the transcriptome. Specifically, an initial oxidative and alkylating stress-related response of the transcriptome during the early phase of TBH treatment was found to be followed by a response that determines cell-survival. Also, we identified genes of which the expression seems directly regulated by DNA methylation. This work suggests an important role of the methylome in counter-regulating primary oxidative- and alkylating stress responses in the transcriptome in order to restore normal cell function.

Introduction

Cancer is traditionally described as a disease induced by accumulations of gene mutations that favor the cell into immortality by stimulating cell growth and by inhibiting apoptosis [1]. Recently, genomic instability has been described as an additional hallmark of cancer which may occur through non-mutational mechanisms such as changes in chromatin structures induced by the deregulation of DNA methylation and histone modifications [2]. DNA methylation of CpG islands is the most widely studied epigenetic phenomenon in cancer. DNA methylation occurs through the enzymatic addition of methyl groups to the fifth position of cytosine, by DNA methyltransferases (*DNMT*), using S-adenosylmethionine (SAM) as the methyl donor. Conversely, these methyl groups can be enzymatically removed by demethylases (or dioxygenases), in particular from the TET enzyme family [3]. Aberrantly high CpG methylation in promoter and exon 1 regions may lead to transcriptional repression and loss of gene function [4-6].

Enzymatic processes of DNA methylation and demethylation, which thus are essential for the maintenance of normal cellular function, may be disrupted by a number of extracellular and intracellular factors. These factors include free radicals such as oxygen radicals (hydroxyl radicals, superoxide anion radicals) and carbon-centered radicals (methyl radicals) [7, 8]. These can be produced as byproducts of intracellular processes as well as induced by extracellular stressors such as oxidative compounds, *e.g.*, hydroperoxides, genotoxic compounds, UV, or ionizing radiation. Failure to activate an adequate antioxidant response via transcription factors such as the nuclear factor (erythroid-derived 2)-like 2 (*NRF2*), may subsequently lead to chronic oxidative stress [9]. The resulting damage leads to accumulation of oxidation products, DNA mutations and chromosomal aberrations, and might thus contribute to tumorigenesis [10]. Oxidative stress-induced mechanisms have for instance been related to chronic liver diseases and hepatocellular carcinoma (HCC) [11]. In recent years, it has been shown that free radicals can directly act on DNA methylation [8]. In this context, it has been proposed that in HCC, free radicals induce transcriptional silencing of tumor suppressor genes, such as suppressor for cytokine silencing 1 (*SOCS1*) and e-cadherin 1 (*CDH1*), by hypermethylation of their promoter regions [12, 13]. In particular, it has been suggested that oxidative stress in HCC induces these alterations in DNA methylation status by affecting the activity of *DNMT* [8, 14]. However, in general, the mode of action by which free radicals impact on the epigenome, remains unclear.

Therefore, in this study, we sought to obtain a better understanding of the response of the methylome following exposure of the liver cell line HepG2 to exogenously generated free radicals, by applying microarray-based technologies. We specifically analyzed the interplay between global gene expression changes and 5-methylcytosine (5mC) status in response to *tert*-butyl hydroperoxide (TBH) exposure.

TBH is metabolized into hydroxyl- and methyl radicals [15], and is thus capable of inducing respectively cellular oxidative and alkylating damage. This may result in S phase arrest, lipid peroxidation, and DNA damage, and may be accompanied by upregulation of the NRF2 pathway and subsequent antioxidant genes [15-17]. Furthermore, using a cell free system, Kasai and Kawai [7] observed that methyl radicals, formed by TBH, randomly attack cytosine residues to form 5mC in DNA with a low yield and in an unspecific manner for CpG sequences.

However, it is not clear whether methyl radicals can directly react with genomic DNA in a cell-system such as HepG2 cells, which contains an active antioxidant machinery and active DNA repair [15] as well as possesses the ability of metabolizing methionine to form the methyl donor, SAM, in combination with high DNMT activity [18, 19]. Therefore, with the aim to obtain new insights into the sequential cross-talk between genetic and epigenetic response in a cellular liver model, we focused on the promoter and exon 1 sites of genes strongly induced in HepG2 cells in response to oxidative and alkylating stress. Temporal exposure analysis (1, 8 and 24 h) demonstrated an early primary antioxidative response of the transcriptome to TBH exposure followed by a later adaptive response of the methylome. Overall, this study provides new insights into the transient oxidative stress-related response in HepG2 cells after TBH treatment controlled by the interplay between gene expression and DNA methylation.

Material and methods

Cell culture

HepG2 cells (ATCC, LGC logistics) were cultured in 6-well plates in the presence of minimal essential medium supplemented with 1% nonessential amino acids, 1% sodium pyruvate, 1% penicillin/streptomycin, and 10% fetal bovine serum (all from Gibco BRL, Breda, The Netherlands). The cells were incubated at 37°C and 5% CO_2. When cells were 80% confluent, the medium was replaced with medium containing 200 µM TBH (Sigma-Aldrich, Zwijndrecht, The Netherlands) and exposed for 1, 8 and 24h. As a solvent control, medium was used. Time-matched control cells were treated in an identical manner without addition of TBH.

Identification and levels of radical formation

Radical formation in HepG2 cells was measured by ESR spectroscopy in combination with the spin trapping technique using 50 mM 5,5-dimethyl-1-pyrolline N-oxide (DMPO, Sigma-Aldrich, Zwijndrecht, The Netherlands) as described previously [20].

Chemical identification of 5-methylcytosine DNA adducts

After DNA isolation and digestion using NaI and P1 nuclease as described before [21], a HPLC system (Shimadzu LC-10AD pumps, DGU-10A degasser, SIL-HTc autoinjector, CTO-10AS column heater, SPD-20A UV detector) coupled to a

tandem mass spectrometer (MS/MS; API 3000 with Turbo Ionspray; AB-Sciex) was used to analyze modified nucleotides. The products were separated using an octadecylsilicagel (ODS) column (250 length x 2.0 mm I.D. particle size =5um; YMC) and eluted with a gradient buffer solution (5 mM formate buffer, pH 5) with increasing acetonitrile (Optima, Fisher Scientific) from 0% to 30% in 15 min at a total flow rate of 0.2 mL/min. The duration of analysis was 30 min, which included the gradient program for separation (15 min), a column wash with 70% acetonitrile (10 min) and equilibration with initial buffer solution (5 min). The eluent was split to the MS/MS instrument (~85%) and the UV detector (~15%). The products were detected by MS using positive ionization using multiple reaction mode (MRM) with optimized mass to mass transitions and collision energies for each nucleoside. MRM analysis included the appropriate transitions for natural and isotopically-labeled compounds and the yield of products was determined from the ratio of ion signals for the natural and corresponding isotopic standards (+2 or +3 a.m.u.) (Supplementary data 1). The amount of DNA injected was estimated by UV detection using a standard solution of nucleosides. A sample containing a known amount of calf thymus DNA was run in parallel with extracted DNA samples to correct for variations in the efficiency of digestion, the separation, UV absorption and MS detection.

Total RNA isolation and whole genome gene expression

Total RNA was extracted using 0.5 ml QIAZOL (Qiagen, Westburg, The Netherlands) according to the manufacturer's instructions. MiRNeasy Mini Kits (Qiagen, Westburg, The Netherlands) were used to purify total RNA. cRNA was prepared using Affymetrix synthesis and labeling kits as described before (Affymetrix, Santa Clara) [22]. cRNA was hybridized on high-density oligonucleotide GeneTitan chips (Affymetrix Human Genome U133 Plus PM GeneTitan 24 arrays) as described before [15].

Purification of 5mC enriched DNA fragments

Cells were collected in digestion buffer (1mM EDTA; 50mM Tris–HCl, pH 8.0; 5% SDS) and proteinase K (1mg/ml) (Ambion, Bleiswijk, The Netherlands). 1:1 phenol-chloroform-isoamylalcohol (PCI) (Sigma-Aldricht, Zwijndrecht, The Netherlands) was added and the upper phase was precipitated using 3M NaAc pH 5.6 and cold 100% ethanol.

Genomic DNA was fragmented to range between 200bp to 600bp and purified using silica columns (Zymo Research, Freiburg, Germany) and eluted in TE buffer. MeDIP was performed using the MagMeDIP kit (Diagenode, Liege, Belgium) according to the manufacturer's protocol. Briefly, IP incubation mix was added to 1.2 µg sonicated DNA sample and denaturated at 95°C. 10% of this was used as reference samples. The remaining sample was immunoprecipitated overnight using antibody mix containing the 5-methylcytidine antibody and magnetic beads. Following purification using the Ipure kit (Diagenode) according to manufacturer's protocol, reference and MeDIP samples were prepared for

microarray analysis by whole genome amplification (WGA) using the WGA2 kit (Sigma-Aldrich) as described by the manufacturer's protocol without performing the fragmentation step. Methylation enrichment in the paired samples MeDIP/reference was derived from qPCR data by calculating the ratio positive control/negative control, applying the ΔΔCt method.

MeDIP-Chip

For whole genome analysis of DNA methylation levels, the Human DNA Methylation 2.1M Deluxe Promoter Array (Roche NimbleGen, Basel, Switzerland) was used. This type of platform has already been applied successfully in previous *in vitro* and *in vivo* studies [23, 24]. These arrays have a density of 2.1 million probes (50-75 oligonucleotides long, median probe spacing 100 bp) that represent all annotated human promoters (~ 26,210), 27,867 CpG islands, and 750 miRNA promoters per slide. Labeling and hybridization of arrays was performed according to the manufactures' protocol. Briefly, reference and MeDIP DNA were labeled with Cy3 and Cy5 respectively by random priming using the Dual Color DNA labeling kit (Roche NimbleGen) and hybridized using the NimbleGen hybridization kit (Roche, NimbleGen). Samples were hybridized overnight on the 2.1M Deluxe Promoter Arrays using the HX1 mixers and the NimbleGen Hybridization system 4. Slides were washed using the NimbleGen wash buffer kit and scanned using the 2µm high resolution NimbleGen MS 200 micro array scanner.

Whole genome gene expression data analysis

Data normalization and filtering

Data from eighteen arrays were obtained (samples in biological triplicate for three time points/treated and untreated (=3x3x2=18) HepG2 cells), and Robust Multi-array Average (RMA) normalized and re-annotated to custom CDF files (version 14.1) using the arrayanalysis tool (http://arrayanalysis.org/). Using the bioConductor package LIMMA version 3.18.3 [25], differentially expressed genes (DEGs) were determined. A linear model was fitted to the gene expression data whereby replicate information (paired) was treated as random effect. Next, contrasts were defined that estimated the effect of TBH over medium controls. A moderate t-test was computed and corrected for multiple testing (FDR < 0.05). Using this statistic approach, we were able to obtain DEGs coherently with the stringent identification of differentially methylated regions (DMR), as described later.

The data discussed in this publication have been deposited in NCBI's gene expression omnibus [26] and are accessible through GEO series accession numbers:
GSE39291: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE39291
GSE63989: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE63989

Pathway analysis

ConsensusPathDB [27] was used to identify and visualize the involvement of the DEGs and biological processes that may be affected at the level of pathways, by selecting significant

Heatmap analysis of gene expression regulation over time

Log2 ratios of gene expression at 3 different time points (1, 8 and 24h) of selected genes after TBH treatment in HepG2 cells were visualized using a heatmap creating script in Bioconductor R.

DNA methylation data analysis

Normalization and log2 ratio calculations

Signal intensity data was extracted from the scanned images of each array using NimbleScan v2.6 software and quantile normalized on a per channel basis. Log2 ratios of the intensities were computed (ratio of MeDIP signal / reference signal) and for each array, centering was performed by subtracting the global array bi-weight mean of the log2 ratios such that the computed log2 ratios were centered around 0.

Sliding window-ANOVA to define differentially methylation regions

Identification of differentially methylated regions was carried out as described previously with minor modification [24]. Detection of differential methylation was performed by applying the Probe Sliding Window-ANOVA algorithm (PSW-ANOVA). PSW-ANOVA uses a sliding window approach to identify potential sites of differential enrichment and then assigns probability scores (p values) to each probe on the array using a repeated measure ANOVA model. PSW-ANOVA is implemented in the R statistical programming environment as a custom script developed in collaboration with Roche NimbleGen. Briefly, for each probe, a sliding window is determined based on the sliding window size parameter, centering on the probe in question. PSW-ANOVA (sliding window of 750 bp comprising 7 probes, and a FDR adjusted p- value < 0.05) was used to identify DMR which were significantly different between the conditions tested in the experiment (*e.g.*, control vs exposed). Peaks were identified in the DMR, using highly stringent criteria with respect to the required number of probes, i.e. at least 8 consecutive probes should be statistically significant (p- value < 0.05) in order to form a peak.

These peaks were mapped to promoter regions (from 3 kb upstream to 1 kb downstream of the transcription start site [28]) and CpG islands of genes using the NimbleScan v2.6 software. A control corrected mean log2 ratio was calculated for the peaks mapped on to these genes for each time point. Log2 ratios > 0 indicate hypermethylation and log2 ratios < 0 indicate hypomethylation of the DMR by TBH treatment.

Mapping peaks to different regions in the human genome

In order to map the discovered peaks to regions of the human genome (HG19) in more detail, HOMER [29] was used. AnnotatePeaks.pl was used following the default settings and enabled peak mapping on to promoter, exon, intron, intergenic, 3'UTR, 5'UTR and transcription termination sites (TTS).

q-PCR validation of array results

Validation of a selection (based on significance at transcriptome or methylome level) of genes was performed using 10 ng reference and MeDIP sample after WGA (Sigma-Aldrich), as described in the manufacturer's protocol, for quantitative PCR to calculate the ratio of methylation enrichment between exposed/control, applying the ΔΔCt method. Primer sequences, as designed by primer 3 (http://bioinfo.ut.ee/primer3/), are available in Supplementary data 2.

Results

Methyl radicals did not induce a net increase in 5mC levels in TBH-treated HepG2 cells

We hypothesized that TBH-derived methyl radicals serve as a methyl donor for the formation of 5mC in the genome. To investigate this, we first determined the formation of methyl radicals after exposure of HepG2 cells to TBH. TBH was able to produce significant amounts of methyl (DMPO$^{\bullet}$-CH_3) and hydroxyl (DMPO$^{\bullet}$-OH) radicals as measured by ESR spectroscopy after 30 minutes (Figure 1A). We previously showed that these levels decreased to basal levels after 2h exposure and completely disappeared after 24h exposure [15]. Importantly, no methyl radicals were formed in the absence of HepG2 cells, indicating that radical formation upon TBH exposure requires cellular metabolism (Figure 1B).

To investigate whether these alkylating methyl radicals have a direct effect on cytosine methylation globally, we measured DNA methylation in genomic DNA extracted from TBH-treated and untreated HepG2 cells, by using LC-MS/MS. Strikingly, TBH treatment did not affect global genomic 5mC levels at any of the measured time points (1h, 8h and 24h) (Figure 1C).

Altogether, these data clearly demonstrate that TBH-derived methyl radicals, despite being formed in relevant amounts, do not induce a net increase in 5mC level across the genome.

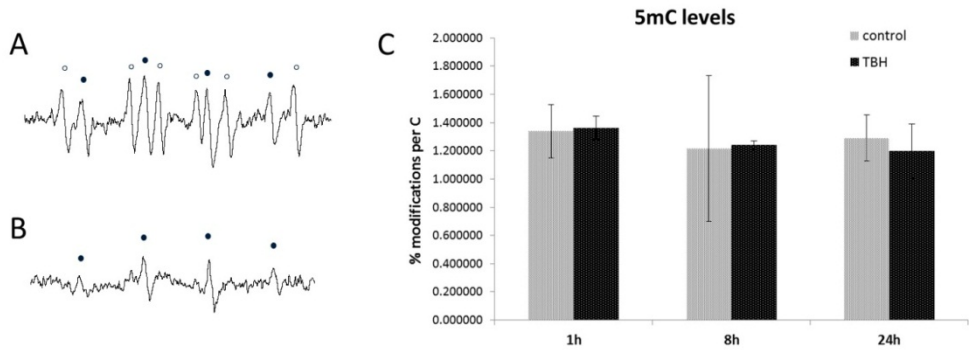

Figure 1 (A) Spectrum of methyl (DMPO•-CH_3, indicates as: ○) and hydroxyl (DMPO•-OH, indicated as: ●) radicals as measured by ESR spectroscopy after 30 minutes TBH treatment in the presence of HepG2 cells. (B) Spectrum of 30 minutes TBH treatment in the absence of HepG2 cells, which only produced hydroxyl radicals (DMPO•-OH, indicated as: ●). (C) Levels in percentage of 5mC adducts per cytosine in complete DNA of TBH-treated and untreated HepG2 cells as detected by LC-MS/MS (n=3, $p<0.05$).

Gene-specific methylation- and gene expression changes

Distribution of 5-methylcytosine modifications induced by TBH

Next, we investigated whether TBH exposure mediates specific changes in genomic 5mC patterns. To this end, we performed methyl-DNA immunoprecipitation (MeDIP) followed by detection of methylated regions by applying high density promoter microarrays. This data was compared with previously obtained whole genome gene expression data [15] and plotted to compare DNA hyper- and hypomethylation (Figure 2A) versus up- and downregulation of genes (Figure 2B), following TBH treatment.

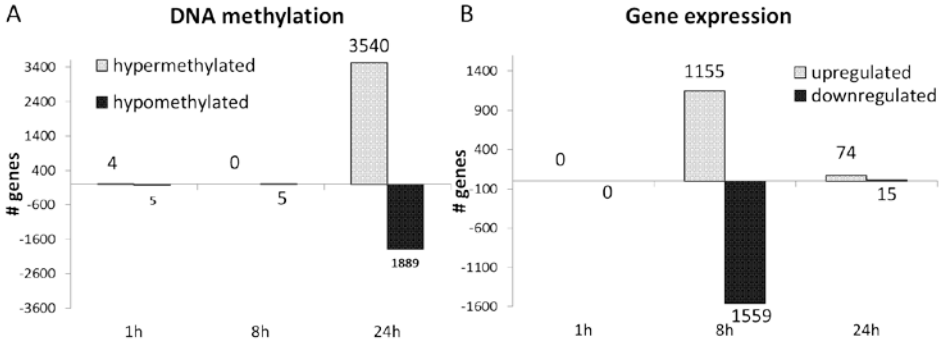

Figure 2 (A) Number of genes that have significant hyper- or hypo-5mC level changes after 1, 8 and 24h in HepG2 cells. Differentially methylated regions were obtained using the sliding window-ANOVA approach and mapped to genes using human genome 19. (B) Number of genes that were differentially upregulated or downregulated as a response to TBH exposure during 1, 8 or 24h in HepG2 cells. DEGs were obtained using a FDR corrected p-value obtained by Limma ($FDR<0.05$, $p<0.05$).

The methylome showed a massive response at 24h of TBH treatment resulting in 5227 differentially methylated genes (DMGs) of which 1889 hypo- and 3540 hypermethylated genes (Figure 2A). By contrast, almost no effect was observed following 1h (hyper: 4 DMGs; hypo: 5 DMGs) and 8h (hyper: 0 DMGs; hypo: 5 DMGs) of TBH challenge (Figure 2A). The majority of these 5mC level changes after 24h of TBH treatment was localized within 2.5kb downstream and 2.5kb upstream from the transcription start site (TSS) of genes (Figure 3A). Changes were mainly observed within the gene body (intron, exon, UTR and TTS regions, 49%) followed by promoter and exon 1 regions (34%). 17% of altered 5mC status was observed as intergenic (Figure 3B).

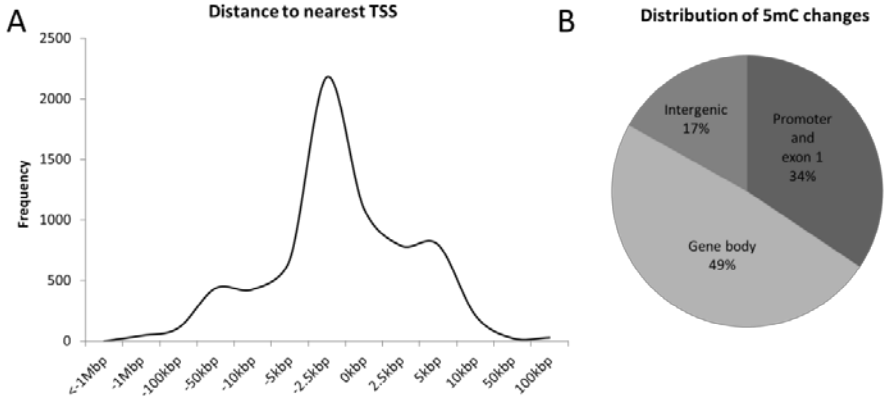

Figure 3 (A) A histogram of the distance of differentially methylated regions after 24h TBH treatment to the transcription start site (TSS) as obtained by Homer. (B) Pie chart distribution of significant 5mC changes in promoter and exon 1, gene body and intergenic.

Since hypermethylation of the promoter and exon 1 region is directly linked to transcriptional silencing, we were mainly interested in these regions. Among many others, oxidative stress-related genes, NAPDH oxidase (*NOX5*), FBJ murine osteosarcoma viral oncogene homolog (*FOS*), v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog genes (*MAFB*, *MAFG* and *MAFK*) (Figure 4A, B and C) and heme oxygenase 2 (*HMOX2*) (Figure 4D), were hypermethylated in the promoter or exon 1 region after 24h of TBH treatment. In addition, different tumor protein p53 (*TP53*)-regulating genes demonstrated modifications in 5mC patterns, such as *TP53BP1* and homologue tumor protein p73 (*TP73*) (Figure 4A, B and C).

Validation of changed 5mC status using quantitative-PCR

After identification of these different DMGs, an independently conducted validation of the array analysis results was performed by means of qPCR analysis of samples of HepG2 cells treated with TBH for 24h. In each investigated case (*CBS, BHMT, FOS, HMOX2, TP53BP1, LIF, RYBP, NUPL1, SRXN1, AKT2, MAFB*), the regions defined as losing or gaining methyl groups, were successfully validated (Figure 4E).

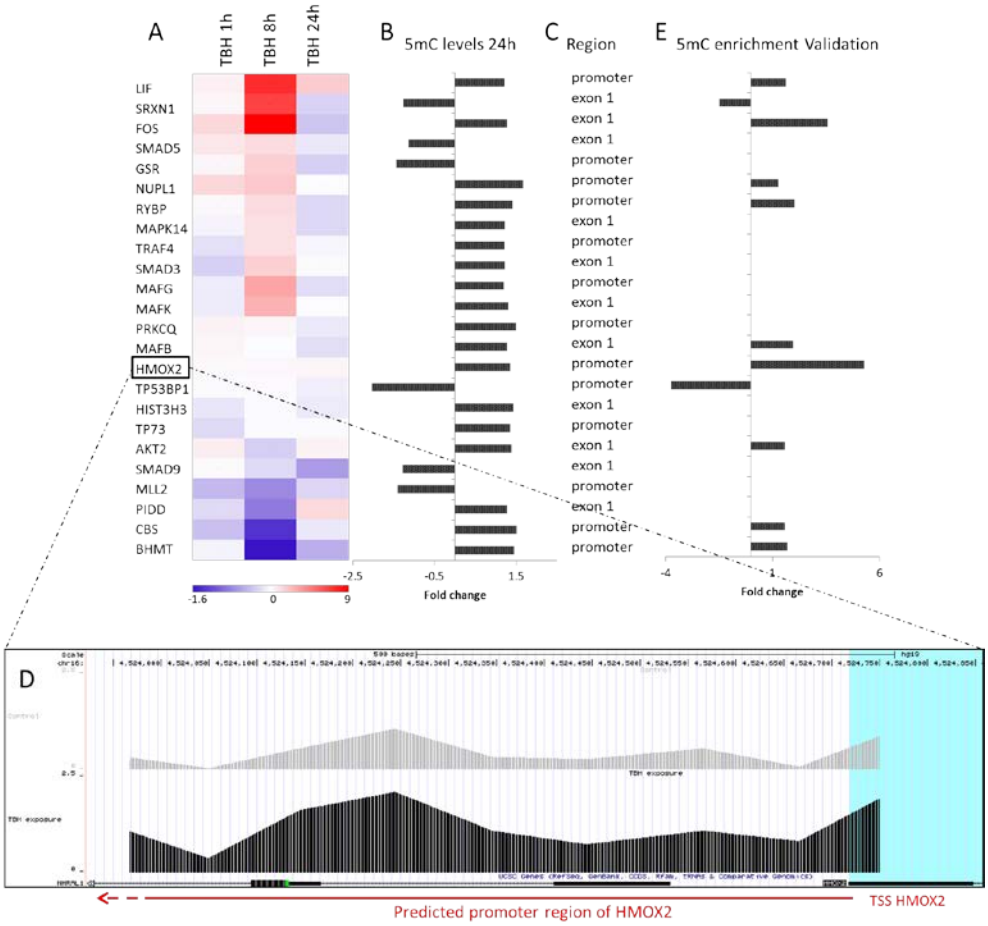

Figure 4 Selection of genes with their (A) expression levels after TBH treatment during 1, 8 and 24h presented as fold change compared to control using a heatmap. (B) In addition, 5mC levels after 24h were presented in fold change values accompanied by the (C) region where these changes took place. (D) This was visualized for HMOX2 where the peak as identified using a window sliding-ANOVA was observed in the predicted promoter region of this gene and was increased after TBH treatment compared to control. (E) An independent validation of the array analysis results was performed using 24h TBH treated samples with qPCR. In each case the regions defined as losing or gaining methyl groups were successfully validated.

Oxidative stress-related responses of the transcriptome induced by TBH treatment

In contrast with the methylome, the transcriptome appeared differentially induced especially after 8h of oxidative and alkylating challenge. This resulted in 1155 upregulated and 1559 downregulated genes (Figure 2B). These differentially expressed genes (DEGs) were involved in cell cycle processes, telomerase activity, DNA strand elongation, cellular senescence, DNA damage repair, oxidative stress and DNA polymerase activity (Supplementary data 3). Among others, important oncogenes/transcription factors, *FOS*, *JUN*, *MDM2* and *MYC*, were upregulated

following 8h of TBH exposure in HepG2 cells as well as oxidative stress-related genes *NRF2*, *HMOX1*, nuclear factor kappa b (*NF-κB*), mitochondrial superoxide dismutase 2, *(SOD2*), sulfiredoxin 1 (*SRXN1*), glutathione reductase (*GSR*) and glutamate-cysteine ligase, catalytic subunit (*GCLC*). 5-methyltetrahydrofolate-homocysteine methyltransferase (*MTR*), serine hydroxymethyl-transferase 1 (*SHMT1*) and choline dehydrogenase (*CHDC*), all involved in the formation of methyl donor, SAM, were downregulated while adenosylmethionine decarboxylase 1 (*AMD1*), involved in SAM carboxylation was upregulated after 8h of TBH treatment. Expression of methyltransferase genes (*DNMT1, DNMT3A, DNMT3B*), was not differentially changed following TBH exposure.

After 24h treatment, the amount of changes in gene expression decreased to only 89 DEGs, of which 74 genes were upregulated and 15 were downregulated (Figure 2B). For instance, a loss of significantly increased NRF2 expression and its downstream responsive genes was observed after 24h of TBH treatment. Additionally, the observed downregulation of *MTR*, *SHMT1*, *CHDC* and *AMD1* after 8h normalized to control expression levels after 24h of TBH challenge.

On the other hand, target genes from TP53 such as fas cell surface death receptor (*FAS*), p53-induced death domain protein (*PIDD*) and ferredoxin reductase (*FDXR*) were upregulated after 24h of TBH challenge as well as cyclin-dependent kinase inhibitor (*CDKN3*), essential meiotic structure-specific endonuclease (*EME1*) and cytoglobin (*CYGB*). These genes may thus be activated as a response to cellular damage such as S phase arrest and oxidative DNA damage, as observed in a previous study [15] after 8h of TBH treatment.

In total, these results demonstrate that the oxidative stress-related gene expression response after 8h of exposure is normalized after 24h of TBH challenge.

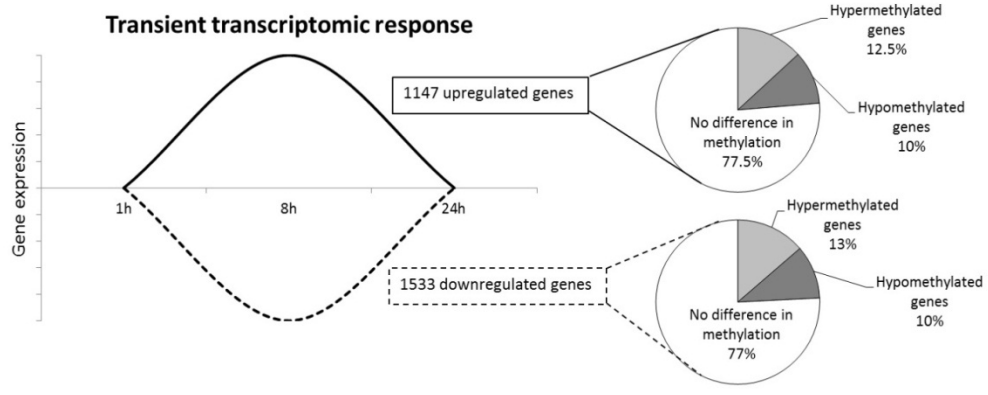

Figure 5 Transient response of the transcriptome induced by TBH challenge including up- and downregulated DEGs. The amount of genes containing hypo- and hypermethylated promoter or exon 1 regions was given for both upregulated and downregulated genes in a pie chart.

Control of gene expression by DNA methylation

Figure 5 presents the transient gene expression response after TBH treatment in general. For all DEGs which were upregulated after 8h of TBH treatment, 12.5% were observed to be hypermethylated in the promoter or exon 1 region at 24h of exposure. Interestingly, these counter-regulated genes were involved in pathways such as oxidative stress-induced gene expression by *NRF2*, glutathione synthesis, receptor activator of NF-kappa-B ligand (*RANKL*), and tumor growth factor beta (*TGFβ*) signaling. On the other hand, 10% of all upregulated genes following 8h of TBH exposure, showed hypomethylated promoter or exon 1 regions after 24h. Pathway analysis identified processes such as apoptotic pathways, ribosome biogenesis and T-cell receptor signaling. Expression levels of these genes, however, despite still being hypomethylated, reached control levels after 24h of treatment.

Similarly, from all transiently downregulated genes, 10% had hypomethylated promoter or exon 1 regions after 24 h of exposure. This counter-regulated the repression of these genes which had thus reached baseline expression levels again after 24h of TBH treatment. These genes were mainly involved in cell cycle signaling and DNA strand elongation. In addition, we also observed that 13% of all genes downregulated at 8h of TBH exposure, showed hypermethylated promoter or exon 1 regions after 24h treatment. This, however, did not lead to further repression of gene expression. These hypermethylated genes were especially involved in pathways such as fibroblast growth factor, methionine salvage and insulin receptor signaling.

These results show that gene expression changes induced by TBH were mainly transient over time and were only partly regulated by the methylome. Especially the repression of oxidative stress-responsive genes towards TBH treatment was regulated by the methylome. This is exemplified in the next section with regard to the NRF2/KEAP1 pathway.

Interplay of the transcriptome and methylome within the NRF2/KEAP1 pathway

Next, we zoomed in on the NRF2/KEAP1 pathway which is considered of crucial importance in the regulation of antioxidant responses.

As already described, an early activation of NRF2 and its downstream genes at the transcriptome level was observed (Figure 6A), while genes involved in the regulation of NRF2-activation and further antioxidant genes appeared hypermethylated in their promoter or exon 1 region (Figure 6B) after 24h of treatment. In more detail, in an early response to TBH, gene expression of *MAPK14* and *JUN* was transiently increased (Figure 6A) and may therefore induce NRF2 activation. This was followed by hypermethylation of promoter or exon 1 regions of these genes after 24h of TBH treatment (Figure 6B), leading to gene expression levels that reach control levels. In contrast, protein kinase C (*PRKCE*) expression was transiently downregulated (Figure 6A) after 8h of TBH treatment and reached control levels after 24h of exposure and was therefore probably not responsible for NRF2 activation. Co-activators, *MAFK* and *MAFG*, which proteins

bind with NRF2 at the antioxidant response element, were upregulated after 8h of TBH treatment (Figure 6A) followed by hypermethylation in their exon 1 and promoter region respectively (Figure 6B). This resulted in a transient activation of downstream response genes, *HMOX1*, *GLCL* and *GCLM*. Based on these results, we conclude that activation of NRF2 and its downstream genes, present at 8h TBH exposure, was generally counter-regulated after 24h by the methylome, as inferred by inactivating upstream genes and co-activators.

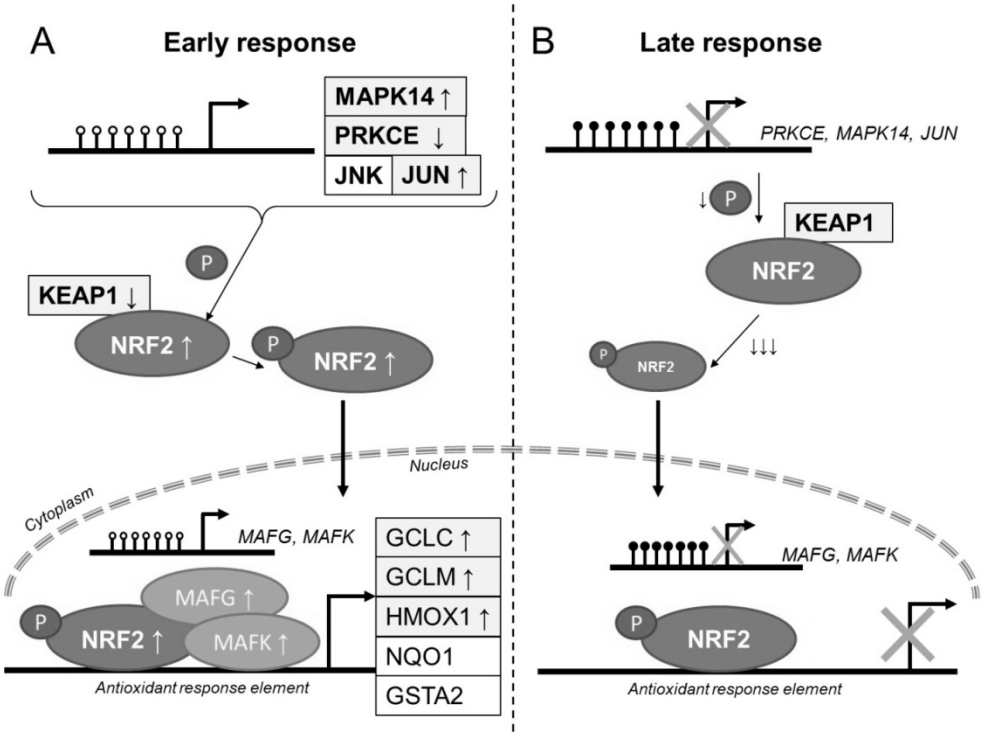

Figure 6 Schematic overview of NRF2 activation to induce phase II response genes by transportation to the nucleus and binding to the antioxidant responsive element. (A) An early response to TBH exposure (8h) which upregulates expression of different kinases (*MAPK14*, *JNK*/*JUN*, *PRKCE*) to phosphorylate NRF2. NRF2 binds together with small maf to the antioxidant responsive element to induce increased expression of phase II responsive genes (upregulation: ↓, downregulation: ↑). (B) During a late response to TBH treatment (24h), hypermethylation of these kinases was observed as well as hypermethylation of *MAFG* and *MAFB* which represses expression of phase II responsive genes over time.
○= non-methylated promoter/exon 1 region ●= methylated promoter/exon 1 region

Discussion

It is well established that oxidative stress and exposure to methyl radicals elicits a cellular adaptive response by activating a transcriptional program [7, 14]. However, our current understanding on how this transcriptional response is regulated remains limited. Recently, it was shown in a cell-free system that methyl

radicals can directly serve as methyl donors for the formation of 5mC from cytosine residues in the DNA backbone [7]. This raised the hypothesis that in the intact cell, DNA methylation plays a substantial role in regulating the transcriptional response after exposure to methyl radicals. To address this hypothesis, we investigated the effects of methyl and hydroxyl radicals formed during TBH exposure on the methylome in HepG2 at multiple time points (1, 8 and 24h). The cellular formation of these radicals was confirmed by ESR spectroscopy.

Our results reveal that methyl radicals formed after TBH treatment do not directly attack cytosine residues in the genome of intact cells. We have 4 lines of evidence that support this conclusion; (i) we observed no global increase in 5mC adducts by LC-MS/MS after exposure of HepG2 cells to TBH, (ii) methylome analysis using MeDIP arrays showed both hyper- and hypomethylated regions, (iii) changes in 5mC detected by MeDIP occurred relatively late, which is inconsistent with a radical reaction that takes place immediately, (iv) changes in 5mC occurred in specific genomic regions and not in a random fashion as expected with a radical reaction. Therefore, we suggest that this response of the methylome is regulated by the cell to allow for recovering from oxidative stress-related damage in the form of oxidative and alkylating DNA damage, S-phase arrest and gene expression changes [15].

Our study demonstrates a transient gene expression of 2680 DEGs at 8h exposure. Oxidative stress-related responses at 8h of TBH exposure appeared to be negatively regulated and followed by expression of genes such as *CDKN3*, *EME1* and *CYGB* which are involved in normal mitosis, maintenance of genomic stability and protection against oxidative stress respectively [30-32]. Furthermore, expression of different p53 regulated genes such as *FDXR*, *PIDD* and *FAS*, which have a pro-apoptotic function [33], were upregulated following 24h of TBH treatment. In addition, formation of SAM appears to be negatively regulated by downregulation of *MTR*, *SHMT1* and *CHDC* and upregulation of *AMD1* after 8h exposure while this repression normalizes after 24h of TBH treatment. Although methyl radicals formed by TBH do not appear to be able to directly form 5mC, we suggest that formation of SAM is repressed due to the presence of these methyl radicals.

As mentioned before, aberrant epigenetic changes, and in particular histone modification, are known to contribute to genomic instability. Interestingly, several genes involved in histone methylation including histone-lysine N-methyltransferase (*SETDB1*), chromatin assembly factor 1 (*CHAF1A*), chromobox homologs 2 and 8 (*CBX2, CBX8*), euchromatic histone-lysine N-methyltransferase 2 (*EHMT2*) and histone-lysine N-methyltransferase (*EZH2*), were downregulated, whereas the histone demethylation gene, lysine (K)-specific demethylase 6B (*KDM6B*), was upregulated (Supplementary data 4). These expression changes of histone modifying genes were only observed after 8h of TBH treatment, indicating that expression levels reached basal expression levels after 24h of treatment.

Correlation analysis of genes which demonstrated an overlap of changed methylation with their gene expression resulted in the identification of genes such as *MAFG*, *MAFK*, *MAPK14*, tumor necrosis factor receptor superfamily member

10A (*TNFRSF10A*), *TRAF4*, *LIF*, cell death-inducing DFFA-like effector b (*CIDEB*) and tripeptidyl Peptidase 1 (*TPP1*). These genes were first up- respectively downregulated as a response to 8h of TBH treatment, followed by hyper- respectively hypomethylation at 24h of the promoter regions of these genes which was accompanied with changed gene expression levels reaching control levels again. Both *TRAF* and *TNFRSF10A* are involved in the TNF receptor super-family and are associated with signal transduction of cellular proliferation/differentiation by ERK/MAPK signaling and apoptosis respectively [34, 35]. Elevated expression over time due to hypomethylation of *CIDEB* may result in increased signals which induce apoptosis [36], while upregulation of *TPP1* expression is associated with increasing telomere length and DNA damage repair activity [37]. Therefore, it is suggested that these oxidative stress-related responses after 8h of treatment are followed by a response that determines cell fate after 24h of TBH exposure.

Pathway analysis points to the importance of oxidative and alkylating damage-related pathways such as NRF2/KEAP1, DNA damage responses and glutathione synthesis which were negatively regulated over time by the methylome. This appears from the observation that different MAF genes (*MAFB, MAFG and MAFK*), as well as *MAPK14*, *JUN*, *FOS* and *NOX5* were hypermethylated in the promoter or exon 1 region accompanied by repressed gene expression over time. *MAPK14* on the other hand, is known to be activated in response to oxidative stress and to initiate the transcription of antioxidants and affect cell survival by NRF2 activation [38]. Furthermore, *MAPK14* is also capable of regulating the expression of *LIF*, which in turn activates the JAK/STAT signaling pathway to induce transcription of a wide range of genes [39]. Proteins of *MAFK* and *MAFG* genes belong to the small maf protein family and are essential cofactors of the NRF2-induced activation of the antioxidant response element [40]. Protein of *MAFB*, on the other hand, is found to form heterodimers with proteins of *FOS* and *JUN* to control apoptosis and survival by activation of *TP73*, a tumor suppressor gene responsible for cell cycle arrest [41] which is hypermethylated in the promoter region without affecting expression levels. Also *NOX5*, which protein can generate intracellular superoxide anion radicals, is hypermethylated in the promoter region accompanied by downregulation of expression over time, probably to avoid additional ROS-induced cellular damage. After 24h of TBH treatment, genes involved in glutathione synthesis were diminished to normal levels after an upregulation of related genes at 8h. It is likely that downregulation of *GCLC*, *GCLM* and *GSR* over time was induced by the observed negative regulation of the NRF2 pathway.

Interestingly, *MAPK14*, *TP73* and *NOX5* were also found to be upregulated in HCC [42]. This may indicate that oxidative stress initially promotes a procarcinogenic phenotype after 8h of TBH exposure which is then counter-regulated by the methylome after 24h of exposure to induce normal cell behavior.

In conclusion, comparing the transient gene expression after TBH treatment with changes of 5mC levels in the complete genome has led to a better understanding of TBH-induced transient oxidative stress-related responses. Our

results indicate that TBH-induced methyl radicals are not able to directly function as methyl donors for cytosine bases in the DNA of HepG2 cells. Moreover, changes in DNA methylation are found to take place as an adaptive response towards the cellular stress caused by TBH-derived methyl and hydroxyl radicals. This is indicated by the involvement of various genes and pathways which are associated with the regulation of primary oxidative stress-related responses, and which are negatively regulated by DNA methylation. However, as visualized in Figure 4, not all methylation changes correlate to gene expression changes, implying that additional cellular processes *e.g.*, phosphorylation, acetylation or hydroxylation of genes or histones, may act in concert with methylation, in order to maintain normal gene expression in response to oxidative and alkylating stress.

Supplementary data

You can download all supplementary data using following URL:
https://mega.co.nz/#F!Kp9lmRBA!LUXmdq7NkLOYBfMBOK4njg

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CHAPTER 6

Oxidative stress induces dynamic changes in 5-hydroxymethylcytosine at random

In preparation

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Abstract

DNA methylation is a dynamic process regulated by active methylation and demethylation, and plays a crucial role in biological processes and carcinogenesis. The formation of 5-hydroxymethylcytosine by oxidation of 5-methylcytosine (5mC) has been proposed as an intermediate of active demethylation. However, whether and how this active demethylation is regulated by oxidative stress-related processes is not well understood. Here, we investigate whether free oxygen radicals are capable of directly forming 5hmC and how this impacts on the whole genome. In this study, we initially applied LC-MS/MS technology for the chemical identification of four different oxidation products of cytosine and thymine in HepG2 cells exposed to hydroxyl- and methyl radicals, formed by *tert*-butyl hydroperoxide (TBH), at multiple time points. We observed that TBH is able to induce a significant change in 5hmC and oxidized thymine levels. Next, by further exploring the hydroxymethylome by means of hMeDIP-microarray technology, we observed modifications in promoter or exon 1 regions that are highly dynamic and increased with prolonged oxidant exposure. In general these 5hmC changes are not associated with modified gene expression levels, neither with active demethylation. Therefore, we suggest that this process of 5mC oxidation is induced by the random oxidant attack and therefore, is not regulated. Further research is required to understand the impact of random 5mC oxidation on toxic mechanisms-of-action upon cellular challenge by oxidative stress.

Introduction

Methylation of cytosine to form 5-methylcytosine (5mC) in genomic DNA is a well-established epigenetic mechanism, which mainly takes place at CpG dinucleotides. This epigenetic process is aberrant in different types of cancer, including hepatocellular carcinoma (HCC), where hypermethylation especially occurs in CpG islands and more specifically in promoter regions of tumor suppressor genes [1, 2]. The establishment and maintenance of 5mC depends on DNA methyltransferases (DNMTs) that transfer a methyl group from S-adenosylmethionine (SAM) onto the 5-carbon of cytosine [3]. Removal of this mark occurs through passive or active processes. The Ten-Eleven Translocation (TET) enzymes are capable of oxidizing 5mC to form 5-hydroxymethylcytosine (5hmC) [4]. This base was first identified in 1972 [5] and originally thought to be an oxidatively generated lesion [6, 7]. Three subtypes of these Fe(II) and α-ketoglutarate-dependent enzymes have been identified (TET1, 2 and 3) [8, 9]. All use oxygen for transferring a hydroxyl group to 5mC in order to form 5hmC [8, 10, 11]. Moreover, TET enzymes are capable of further oxidizing 5hmC into 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), which are suggested to be intermediates in the eventual removal of 5mC [10, 12] (Figure 1). These oxidation products can be actively removed through base excision repair (BER) or passively by replication-dependent depletion [13, 14]. These 5hmC modifications are primarily localized within enhancers and gene bodies and are therefore believed to play, next to 5mC, an important role in gene regulation [15].

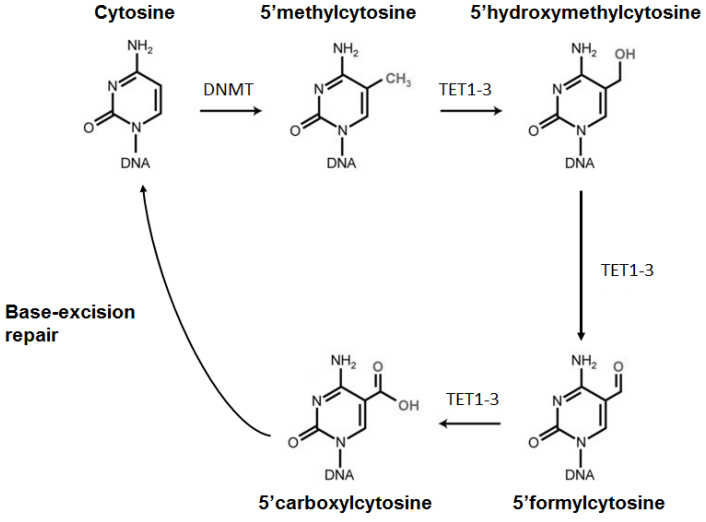

Figure 1 Chemical structure and active conversion of cytosine to 5'methylcytosine by DNMTs, and further oxidized to 5'hydroxymethylcytosine, 5'formylcytosine and 5'carboylcytosine by TET enzymes to form again cytosine after interference of base excision repair. Figure adapted from [Pfeifer, Kadam [16]]. DNMT, DNA methyltransferase; TET1-3, Ten-eleven translocation 1-3 enzymes.

It is well described that oxidative stress may induce major cellular damage over time by attacking multiple cell structures such as lipids, proteins and DNA. Free oxygen radicals can form a multitude of different nucleobase modifications such as 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), which has been described as a hallmark and biomarker of oxidative DNA damage [17]. Furthermore, oxygen radicals are also capable of oxidizing thymine to form 5-formyluracil (5fU) or 5-hydroxymethyluracyl (5hmU) [18, 19]. In recent years, there is considerable debate whether reactive oxygen species may also induce epigenetic alterations, especially by inducing DNA methylation changes and histone modifications (Nishida and Kudo 2013). Interestingly, it has been demonstrated that reactive oxygen species (ROS) can actually induce 5hmC adducts in isolated DNA [6, 20]. However, it is not yet clear whether in a cell model this reaction will occur upon oxidative stress, either directly induced by ROS, or for instance, mediated by TET enzymes.

Overall, the accumulation of these types of oxidative DNA adducts over time, including 5hmC, is observed in different types of cancer, such as hepatocellular carcinoma (HCC), and are considered to play an important role in the alteration of cancer-related genes in the development of HCC [16, 21, 22].

We therefore hypothesize that chemically induced oxidative stress increases 5hmC formation and thereby, elicits a toxic (*e.g.*, carcinogenic) stress response. Thus, we investigated whether *tert*-butyl hydroxperoxide (TBH), a peroxide that forms intracellular methyl- and hydroxyl radicals [23], is able to directly induce 5hmC modifications and how this is time-dependently distributed across the genome. For this, we used HepG2 cells, which possess an active antioxidant machinery and active DNA repair [23, 24] and have been shown to metabolize TBH, thus forming methyl- and hydroxyl radicals [23, 25]. To detect the global dynamic formation of different oxidative adducts in HepG2 cells after short (1h) and longer (8 and 24h) TBH treatment, LC-MS/MS was applied. By comparing these different oxidative pyrimidine adducts (5hmC, 5fC, 5hmU and 5fU), ratios have been calculated to obtain more insights in the approximate level of each substrate in DNA. In addition, we specifically investigated the hydroxymethylome using hydroxymethylated DNA immunoprecipitation (hMedIP)-on-chip technology [26]. The consequences of changed 5hmC levels were investigated by integrating genome-wide DNA hydroxymethylated changes with TBH-induced genome-wide methylation changes and gene expression modifications (as reported in Chapters 2 and 5). This provided us with new information on the consequences of oxidative stress-induced 5hmC changes for gene expression regulation.

Material and methods

Cell culture and treatment

HepG2 cells (ATCC, LGC logistics) were cultured in 6-well plates and treated with the highest non-cytotoxic concentration of tert-butyl hydroperoxide (200µM TBH) (Sigma-Aldrich, Zwijndrecht, The Netherlands) during 1, 8 and 24h as described before (Chapter 5). As a solvent control, medium was used. Time-matched control cells were treated in an identical manner without addition of TBH.

Radical formation, RNA isolation, whole genome gene expression, MeDIP chip, quantitative PCR (qPCR) validation and raw data analysis were performed in an identical manner as described in Chapter 5.

In addition, for 5hmC analysis, sonication-derived DNA fragments were purified similar as 5mC fragments as described in Chapter 5. These DNA fragments were immunoprecipitated by using an antibody mix containing a 5'hydroxymethylcytidine antibody, for 2 hours and adding magnetic beads overnight, according to manufacturer's protocol (Diagenode, Liege, Belgium). 5hmC samples were labeled and hybridized on the Human DNA Methylation 2.1M Deluxe Promoter Array (Roche NimbleGen, Basel, Switzerland) as described in Chapter 5. The obtained signal intensities from these arrays were normalized and significantly hydroxymethylated regions were calculated using the sliding window ANOVA approach, as described in Chapter 5. Peaks in these hydroxymethylated regions were identically identified as in Chapter 5, i.e. a minimum of 8 consecutive probes should be statistically significant (p-value < 0.05) in order to form a peak. Log2 ratios (control corrected) were calculated for the significant peaks (Log2 ratios > 0 indicate hyper-5hmC and log2 ratios < 0 indicate hypo-5hmC in these differentially changed hydroxymethylated regions induced by TBH treatment). Significant peaks were mapped to specific genomic regions using HOMER (Chapter 5).

Chemical identification of oxidation products of 5mC and thymine

After DNA isolation and digestion using NaI and P1 nuclease as described before [20], a HPLC system (Shimadzu LC-10AD pumps, DGU-10A degasser, SIL-HTc autoinjector, CTO-10AS column heater, SPD-20A UV detector) coupled to a tandem mass spectrometer (MS/MS; API 3000 with Turbo Ionspray; AB-Sciex) was used to analyze modified pyrimidines (5-hydroxymethylcytosine, 5hmC; 5-formylcytosine, 5fC; 5-hydroxymethyluracil, 5hmU; 5-formyluracil, 5fU). These products were separated on an octadecylsilyl silica gel (ODS) column (250 length x 2.0 mm I.D.; particle size = 5µm; YMC) and eluted as previously described [20].

Also by means of LC-MS/MS detection, we evaluated the possible artificial introduction of oxidized DNA bases during sample preparation. Therefore, 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) levels were measured as previously described [27]. 8-oxodG levels were measured in untreated HepG2 cells following different sample preparation steps (DNA isolation, sonication and immunoprecipitation) with or without the addition of 0.15mM of the iron chelator

desferroxamine (Sigma-Aldrich, Zwijndrecht, The Netherlands). Antioxidant supplementation did not lead to significant differences in 8-oxo-dG levels (Supplementary data 1), indicating that results obtained did not comprise false positive readouts.

Results

Chemical analysis of DNA adducts formed after TBH treatment

The formation of hydroxyl and methyl radicals in HepG2 cells treated with TBH, was already confirmed in a previous study using ESR spectroscopy [23] (Chapter 2). A significant increase in hydroxyl radical levels was observed after 30 minutes and reached its maximum level after 2h of TBH treatment followed by a decrease to control levels after 4h of TBH treatment. Methyl radicals were only detected after 30 minutes of TBH treatment and were, as discussed in Chapter 5, not able to directly attack cytosine in HepG2 cells.

Also TBH-induced oxidative DNA damage in general, as measured using the FPG comet assay (detection of 8-oxodG and other oxidized purines) [23], was significantly induced after 30 minutes and 1h of TBH treatment followed by a decrease over time to control levels after 2h.

To test our hypothesis that hydroxyl radicals induced by TBH are able to specifically induce pyrimidine oxidation, we measured oxidized cytosine and thymine adducts (5mC, 5hmC, 5fC, 5hmU and 5hU) formation in genomic DNA extracted from TBH-treated and untreated HepG2 cells using LC-MS/MS. Interestingly, MS analysis showed that 8h of TBH treatment led to an overall significant increase in 5hmC adducts compared to control levels and normalized following 24h of exposure (Figure 2A). Furthermore, levels of 5fC, a secondary oxidation product of 5mC, were not significantly increased (Figure 2B). Levels of 5fU, an oxidation product of thymine, were significantly increased after 8h of TBH treatment and normalized after 24h (Figure 2C) while TBH exposure did not lead to changed 5hmU modifications compared to untreated HepG2 cells (Figure 2D). Overall, levels of 5hmC were 5-fold lower than levels of 5fU. In view of the fact that the intracellular ratio of 5mC:thymine is 1:20, we suggest that the net effect of oxidation of 5hmC after 8h of TBH treatment was considerably stronger compared to oxidation of thymine.

By contrast, these increased levels of 5hmC did not result in a global genomic decrease of 5mC levels at any of the measured time points (Chapter 5, Figure 1C).

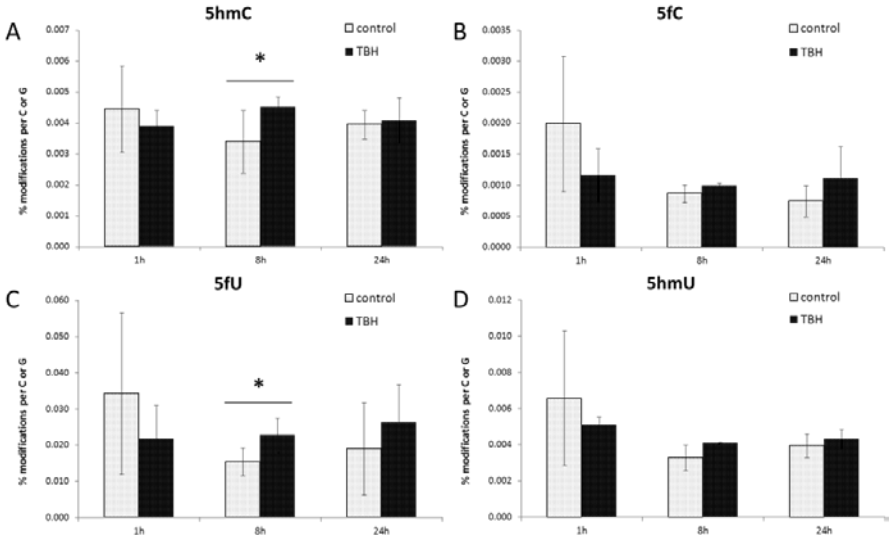

Figure 2 Levels of modifications in percentage/cytosine in complete DNA of TBH-treated and untreated HepG2 cells for (A) 5hmC (B) 5fC (C) 5hmU and (D) 5fU as detected by LC-MS/MS (n=3, $p<0.05$).

Dynamic 5hmC modifications after TBH treatment

To evaluate the genomic distribution of TBH-induced 5hmC changes in more detail, we used hydroxymethylated DNA immunoprecipitation (hMeDIP) in combination with high density promoter microarrays. This technique provided us with the opportunity to visualize gene-specific 5hmC changes following TBH treatment, at multiple time points. When visualizing 5hmC changes which occur after exposure to TBH, we observed a rather unspecific distribution of these significantly hyper- and hypohydroxymethylated regions across the genome, which appeared similar between replicates (Figure 3). A clear difference between treated and untreated conditions can be observed. Moreover, the effect of TBH on the hydroxymethylome tends to increase in correspondence with longer exposure time (Figure 4). When HepG2 cells were exposed to TBH for 1h, 1893 genes showed differences in 5hmC levels compared to control, 48% of these genes containing increased 5hmC levels, and 52% showing lower 5hmC levels (Figure 4A). Treatment during 8h with TBH resulted in a total of 2711 genes that demonstrated changed 5hmC levels. Of all these genes, 49% showed regions with higher 5hmC levels while 51% of these genes had lower 5hmC levels compared to control levels in the same region (Figure 4A). These gene-specific 5hmC modifications increased after 24h of TBH treatment to a total of 6906 genes containing regions with significantly changed 5hmC levels. At this time, approximately 51% of these genes contained regions with higher 5hmC levels and the other 49% contained regions with lower 5hmC levels (Figure 4A) compared to control. When comparing these time-specific 5hmC changes, an overlap for only 263 genes was observed (Figure 4B), suggesting that hydroxymethylation changes induced by TBH across time, were strongly dynamic.

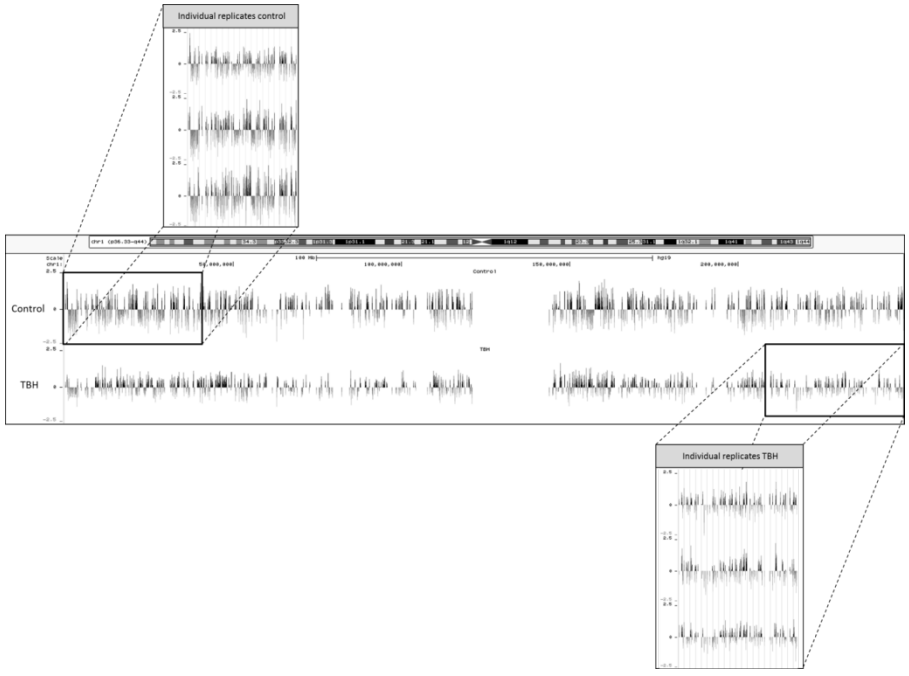

Figure 3 Visualization of significant 5hmC modification patterns after 1h TBH treatment. The average 5hmC pattern at chromosome 1 for untreated and treated HepG2 cells was visualized in combination with separate replicates from the control group and TBH-treated group (n=3, p<0.05). Data is represented by normalized log2 scores identified using a window sliding-ANOVA for all probes across this region. This region was randomly selected and represents the general distribution as observed in the complete genome.

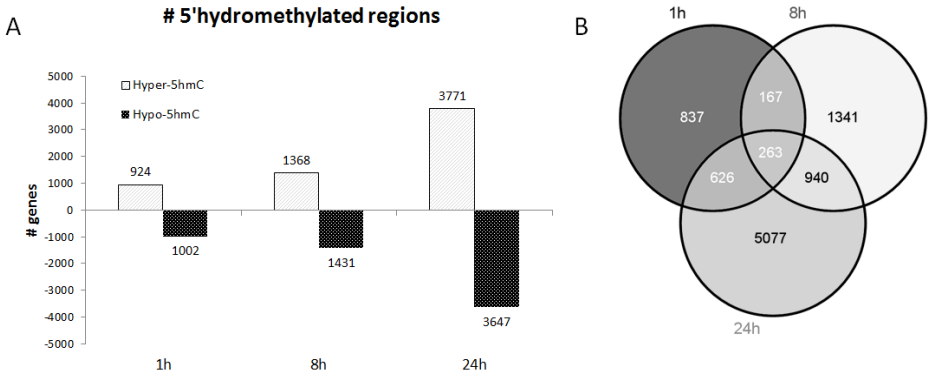

Figure 4 (A) Amount of genes that have significant 5hmC level changes (p<0.05) after 1, 8 and 24h TBH treatment in HepG2 cells. Differentially hyper- and hypo-hydroxymethylated regions were obtained using the sliding window-ANOVA approach and mapped to genes using human genome 19. (B) Venn diagram of genes that contained a significant 5hmC modification during different exposure times to TBH (1, 8 and 24h). In this way unique- and overlapping genes were identified between these different exposure times.

In a next step, to reduce this rather high amount of significantly hydroxymethylated regions, we strengthened the cut-off criteria that were defined for this study (see Methods section of this Chapter and Chapter 5). In particular, the minimal number of statistically significant (p-value<0.05) consecutive probes which defined a "peak", was increased from 8 to 25 probes. This reduced differentially hydroxymethylated gene numbers to only 5, 12 and 105 genes (50% hypo-5hmC; 50% hyper-5hmC) after 1, 8 and 24h of TBH challenge respectively. This data reduction gave us the opportunity to evaluate genes with strongly changed hydroxymethylated regions with respect to their biological relevance in more detail. Pathway analysis of the 105 genes after 24h of TBH treatment did however not result in pathways specifically involved in known oxidative stress responses, nor in signaling pathways for toxic or carcinogenic modes-of-action (data not shown). By contrast, despite this severe data reduction, we still observed a rather unspecific 5hmC distribution that was highly dynamic and increased over time. In order to incorporate all data, we applied the lower cut-off criteria for the remaining data analysis.

Validation of 5hmC changes by qPCR

After identification of these differentially hydroxymethylated regions, an independently conducted validation of the array analysis results was performed on samples of HepG2 cells treated with TBH for 1, 8 and 24h, by using quantitative PCR (qPCR). For each gene (PRKCA, CDK5, TP73, SMAD9, PARP2, MYC, MTRR and WNT5B), the regions defined by microarray as losing or gaining 5hmC groups were, in the majority of cases, successfully confirmed by qPCR (Figure 5).

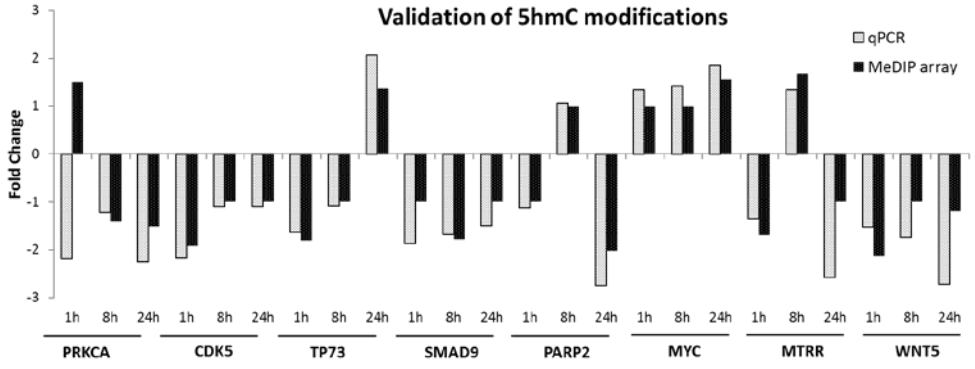

Figure 5 An independent validation of the array analysis results was performed using 1, 8 and 24h TBH treated samples with qPCR. In almost each case the regions defined as losing or gaining hydroxymethyl groups were successfully validated.

5hmC modifications in different DNA regions

It is widely recognized that levels of 5hmC are lower in the proximity of transcription start sites, and are elevated in the gene body [15]. This was also shown by our data, where 5hmC modifications after 1, 8 and 24h of TBH treatment

were mainly localized around 5kb downstream and 5kb upstream from the transcription start site (TSS) of genes, and showed a dip near the TSS (Figure 6A). Moreover, changes in 5hmC levels were especially localized within the gene body such as the intron (45-49%), exon (6-7%), and UTR (2-3%) and TTS (3-4%) as well as were intergenic (22-27%) (Figure 6B). Approximately 12-16% of changes in 5hmC levels were localized within promoter regions at different TBH exposure times (Figure 6B).

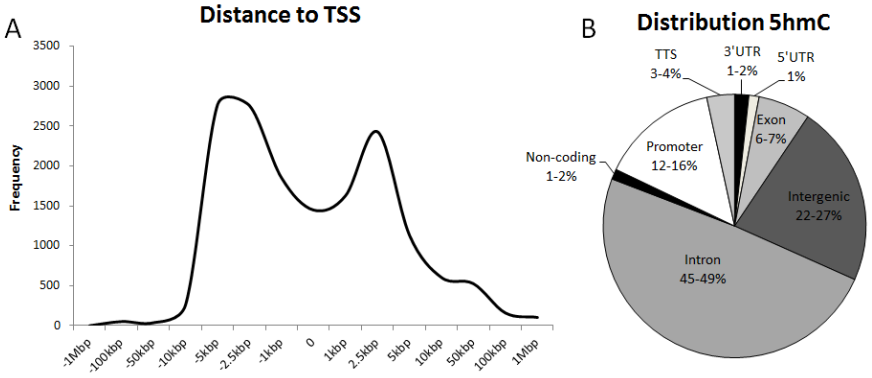

Figure 6 (A) Histogram of the distance of differentially hydroxymethylated regions to the transcription start site (TSS) as obtained by the annotatePeaks.pl sub-program within Homer. (B) Pie chart distribution of the distribution of significant 5hmC changes ($p<0.05$) in different genomic regions: promoter, intron, exon, 5'UTR, 3'UTR, non-coding, intergenic and transcription termination site (TTS) as defined and mapped by Homer using human genome 19.

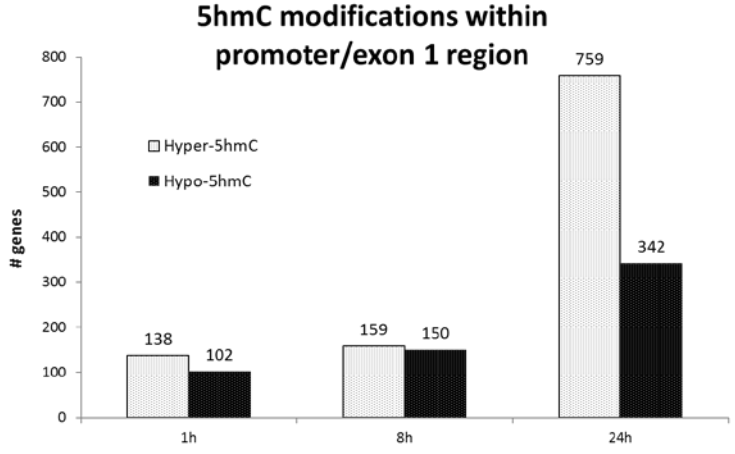

Figure 7 (A) Amount of genes that have significant hyper- or hypo-5hmC levels ($p<0.05$) within promoter and exon 1 regions after 1, 8 and 24h TBH treatment in HepG2 cells. Differentially hydroxymethylated regions were obtained using the sliding window-ANOVA approach and mapped to promoter or exon 1 regions using Homer (Human genome 19).

5hmC modifications within promoter or exon 1 regions

Because transcriptional regulation is especially linked to promoter regions and to the first exon of genes [28-30], we were mainly interested in genes with 5hmC changes in these regions. As previously discussed, we observed that many changes had already occurred after short TBH treatment (1h) indicating that these 5hmC modifications were rapidly induced in response to TBH treatment. In total, 240 5hmC changes (hyper: 138, hypo: 102) were localized within a promoter or exon 1 region following 1h of TBH treatment (Figure 7). Among genes with increased 5hmC levels, glutathione peroxidase 4 and 6, as well as MPG and MBD4, involved in base excision repair (BER), were included. However, pathway analysis of these 240 genes in general did not return biological relevant pathways that could be linked to TBH-induced damage but rather demonstrated very diverse signaling pathways (Table 1A). Following 8h of exposure to TBH, 309 genes (hyper: 159, hypo: 150) were identified to contain significant 5hmC changes in the promoter/exon 1 region (Figure 7). Pathway mapping results again did not relate to previously observed TBH-specific responsive pathways (Table 1B) [23]. These random modifications of the hydroxymethylome even tend to strongly increase after 24h of TBH exposure, comprising 1101 5hmC changes (hyper: 759, hypo: 342) in the promoter or exon 1 region (Figure 7). Once again, pathway analysis of these genes did not identify biologically relevant TBH-responsive processes but showed large signaling pathways instead (Table 1C). We did, however, identify different histone cluster genes involved in the Wnt signaling pathway which is linked to carcinogenesis, to have 5hmC modifications in their promoter or exon 1 region following 24h of TBH treatment. By comparing pathways that were induced following 1, 8 and 24h of TBH exposure, we observed genes involved in G-protein coupled receptor signaling that were shown to contain significantly changed 5hmC levels. The involved genes were, however, different for each time point.

Interestingly, across all time points, we observed more hyper-5hmC regions compared to hypo-5hmC promoter and exon 1 regions, indicating that there is an overall gain in 5hmC modifications in these regions.

Table 1 Significantly regulated pathways based on differentially hydroxymethylated genes in promoter/exon 1 regions. A top 10 of significantly ($p<0.05$) regulated pathways as indicated by ConsensusPathDB is shown for each time point (A) 1h, (B) 8h and (C) 24h of TBH exposure.

	A. Top 10 pathways modified by 1h of TBH treatment	
	Pathway	**P-value**
1	Olfactory transduction	2.52E-05
2	Oxygen-dependent asparagine hydroxylation of HIFα	4.23E-04
3	Olfactory Signaling Pathway	1.80E-03
4	Adrenergic signaling in cardiomyocytes	2.05E-03
5	IL2	2.12E-03
6	Conjugation of salicylate with glycine	2.87E-03
7	NAD Biosynthesis II (from tryptophan)	3.79E-03
8	Conjugation of carboxylic acids	3.79E-03
9	Amino Acid conjugation	3.79E-03
10	GPCR downstream signaling	4.73E-03
	B. Top 10 pathways modified by 8h of TBH treatment	
	Pathway	**P-value**
1	Olfactory Signaling Pathway	6.41E-12
2	Olfactory transduction	1.04E-11
3	GPCR downstream signaling	1.70E-10
4	Signaling by GPCR	1.25E-09
5	Signal Transduction	4.82E-07
6	5-Phosphoribose 1-diphosphate biosynthesis	5.82E-04
7	PRPP biosynthesis	5.82E-04
8	Porphyrin metabolism	1.25E-03
9	Estrogen Metabolism Pathway	1.33E-03
10	Tamoxifen Pathway, Pharmacokinetics	1.89E-03
	C. Top 10 pathways modified by 24h of TBH treatment	
	Pathway	**P-value**
1	Signal Transduction	8.96E-08
2	Signaling by GPCR	2.43E-06
3	Hippo signaling pathway	1.82E-05
4	GPCR downstream signaling	1.65E-04
5	Olfactory transduction	1.96E-04
6	Olfactory Signaling Pathway	2.87E-04
7	Wnt signaling network	3.20E-04
8	GPCR ligand binding	6.98E-04
9	Amine ligand-binding receptors	8.69E-04
10	Dopamine metabolism	2.89E-03

The effect on the transcriptome following dynamic 5hmC modifications induced by TBH

To investigate whether these observed 5hmC changes in promoter and exon 1 regions were functionally linked to changes in gene expression, we compared these results with previously obtained whole genome gene expression data [23]. Rapid dynamic responses of the hydroxymethylome observed after 1h of TBH treatment, did not affect gene expression since no differentially expressed genes were observed at this exposure time. After 8h of TBH exposure, a transient response of the transcriptome was observed, as discussed in Chapter 5. 2714 differentially expressed genes (DEGs) were especially involved in DNA damage responses, cell cycle changes and other oxidative stress-related responses. Interestingly, expression of TET1 at this time point was significantly downregulated. Expression of TET2 and TET3 remained unchanged and was rather low in general in both 8h TBH-treated and untreated HepG2 cells (Supplementary data 2).

Changes in the hydroxymethylome (Table 1) after 8h of TBH exposure, including genes involved in G-protein coupled receptor and specifically Wnt signaling, were not related to these oxidative stress- and DNA damage-related processes apparent at the transcriptome level. Moreover, only 15 of the 2714 DEGs had a changed 5hmC status in their promoter or exon 1. Only 4 of these genes were upregulated after a gain of 5hmC including a nucleic receptor, NR3C1 11 genes were downregulated after a loss of 5hmC, for instance MLL2, which is involved in histone methylation.

The effect of hydroxymethylome modifications on the transcriptome was also limited after 24h of TBH treatment as demonstrated by the small number of DEGs (89 genes) in comparison to the high number of significant 5hmC changes in promoter or exon 1 region (1101 genes). Only 4 DEGs, including cyclin-dependent kinase inhibitor 1A (p21) and glutaminase 2 (GLS2), were identified that had gained a 5hmC modification in their promoter which resulted in upregulation of these genes.

From these results, we conclude that dynamic 5hmC changes in the promoter or exon 1 region had only limited effects on the transcriptome, and overall, tend to be rather randomized.

Loss of 5mC is limited after 5hmC gain in identical genomic regions

Since hydroxymethylation of cytosine hypothetically causes loss of 5mC, irrespectively of which genomic region, by active processes operated by TET enzymes and BER (Figure 1), we were interested to investigate whether these genome-widely 5hmC modifications correlated to changes in 5mC patterns in the same regions. Therefore, we compared hMeDIP with MeDIP results (see Chapter 5). A first observation was the absence of a large response of the methylome after 1 and 8h of TBH treatment while substantial hydroxymethylome modifications were observed during these early exposure times. After exposure of 24h of TBH, on the other hand, 5227 significant 5mC modifications and 6906 significant 5hmC

modifications were assigned to gene-specific regions of which 2406 genes were overlapping (Figure 8A). In most cases (1959, 81%) modifications were observed in different regions within the same gene. From the 449 modifications localized within the same genomic location, 48 (11%) genes showed a gain of 5hmC that was associated with a loss of 5mC. However, this loss of methylation in general did not lead to increased expression of involved genes, except for 2 genes, RAD51C (Figure 8B) and GPRC5B. Also, 5hmC and 5mC modifications were not localized in the promoter or exon 1 region of these genes but in an intron.

Based on the minimal overlap of 5hmC changes with active demethylation and gene expression, we suggest that this process of 5hmC changes induced by TBH, was not a regulated event.

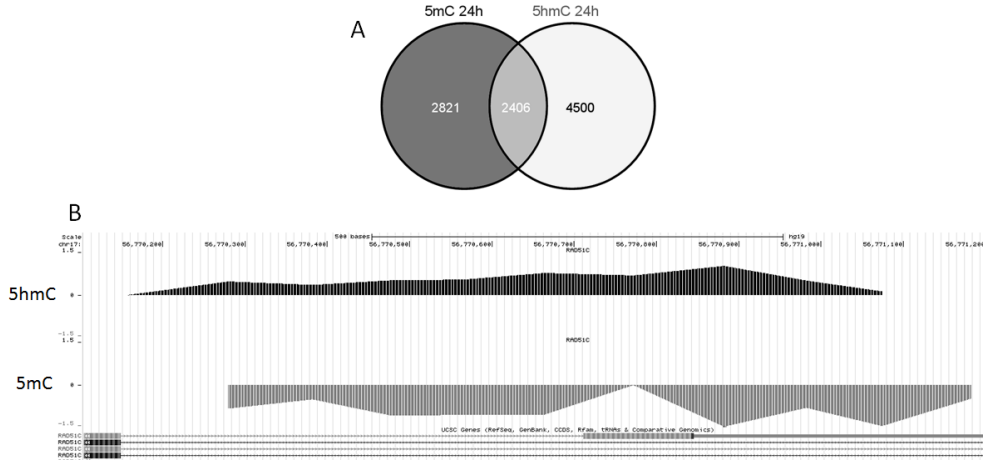

Figure 8 (A) Venn diagram of genes which hold either significant 5hmC or significant 5mC changes ($p<0.05$) after 24h TBH treatment to identify genes that contain both modifications within their DNA sequence during this time point. (B) Visualization of position chr17:56,770,010-56,771,300 (HG19) in UCSC genome browser. 5hmC and 5mC modifications were visualized using the BED graph function and localized within an intron region of RAD51C. Data is represented by normalized log2 scores identified using a window sliding-ANOVA for all significant probes within this 5hmC and 5mC peak.

Discussion

Recent studies have highlighted the importance of 5hmC as an epigenetic mark playing a crucial role in development as well as in different chronic diseases such as cancer [9, 31]. This mark has been characterized as an intermediate of active DNA demethylation by enzymatic mechanisms involving TET enzymes and can therefore induce transcriptional changes [9, 32]. It is well established that oxidative stress elicits such transcriptional changes by interfering with different cellular processes. However, whether oxidative stress can induce perturbed 5hmC distribution in DNA which leads to subsequent changes in gene expression is not clear. Therefore, in this study, we hypothesized that hydroxyl radicals, formed by TBH, are able to directly induce 5hmC formation in HepG2 cells and therefore, we

investigated the genome-wide distribution and downstream effects of oxidative modifications of this DNA base.

While 5hmC levels are known to be relatively low in cultured cell lines [33], in our study, intracellular hydroxyl radicals, formed by TBH metabolism in HepG2, appear to induce a significant increase in 5hmC and 5fU levels after 8h of treatment compared to control, as measured by LC-MS/MS (Figure 9A). The simultaneous formation of oxidation products of both 5mC and thymine after oxidant exposure has already been described previously as being ROS-mediated [20]. We now confirm that the increased formation of 5hmC and 5fU is also more likely induced directly by hydroxyl radicals and not by enzymatic activity of TET enzymes. Although it seems that the net effect of 5hmC oxidation is stronger compared to oxidation of thymine, in total 5hmC modifications are 5-fold lower than 5fU after 8h of TBH treatment, whereas TET-mediated oxidation of 5mC has been associated with much higher 5hmC levels compared to oxidation products of thymine [34]. In addition, after 8h of TBH treatment, we observe a decrease in TET1 expression compared to control expression levels (Figure 9C) while it appears that TET2 and TET3 are not expressed. Depletion of TET1 may lead to a global reduction of 5hmC levels. However, it is possible in our study that TET1 expression is counter-regulated due to the global ROS-mediated increase of 5hmC levels following 8h of TBH treatment.

Interestingly, the formation of these adducts is not increased after 1h TBH treatment, while the formation of hydroxyl radicals and oxidative damage to purines is significantly increased at this time point and reaches control levels again after 8h of TBH exposure (Figure 9A). Possibly, purine oxidation products are removed by DNA repair relatively quickly compared to pyrimidine oxidation products, 5hmC and 5fU, which accumulate and reach significantly higher levels after 8h of treatment (Figure 9A). A difference between these oxidation products is that 5hmC modifications do not miscode during DNA replication. Formation of 5fU by oxygen radicals, on the other hand, has mutational properties and is therefore related to cellular toxicity [35, 36]. However, it has been described that repair of 5fU adducts is slower compared to other DNA adducts [37].

In addition, we have studied the complete 5-hydroxymethylome after TBH treatment by using high density promoter microarrays. This technique has previously been applied in an *in vivo* study in rodents where aberrant 5hmC levels, induced by phenobarbital, are hypothesized to be an underlying mechanism of the development of non-genotoxic carcinogenesis [38]. Short term exposure to phenobarbital (12-24h) does already result in a high number of both hypo- and hyper-5hmC regions which increase with prolonged exposure (7, 28 and 91 days) [26].They observe that elevated 5hmC levels are especially accumulating over a set of induced genes upon challenge by the non-genotoxic carcinogen, phenobarbital [26, 38]. Moreover, they conclude that these particular genes have been described to be transcriptionally perturbed in liver cancers, including human HCC [26]. By defining such regions with differential hydroxymethylation in our

study, we also reveal a high number of these 5hmC changes which increases during prolonged TBH exposure. However, these changes are rather un-specifically and dynamically distributed across the whole genome. Although these 5hmC changes increase with prolonged exposure to TBH (Figure 9B), this does not result in a net increase of 5hmC modifications over time as observed in LC-MS/MS results (Figure 9A). Upon sharpening the cut-off criteria for identifying these significant 5hmC modifications from 8 to 25 consecutive significant probes per peak, this still does not identify pathways related to specific TBH-induced toxicity or to risks for developing HCC.

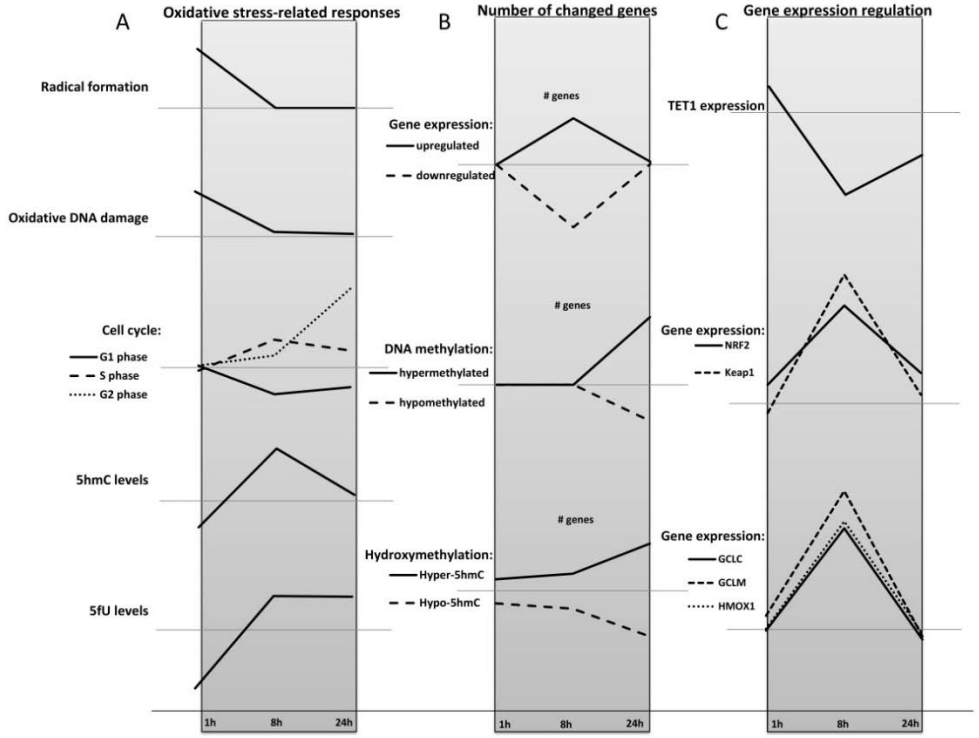

Figure 9 Schematic overview of all TBH-induced cellular changes during 1, 8 and 24h compared to control. (A) levels of hydroxyl radical formation as measured by ESR, oxidative DNA damage as measured by FPG comet assay, cell cycle distribution (G1, S and G2 phase levels) and 5mC oxidation levels in the form of 5hmC formation and thymine oxidation in the form of 5fU levels. (B) The amount of genes that were found to have gene expression changes, methylation changes and hydroxymethylation changes. (C) Schematic overview of gene expression changes of specific genes such as TET1 and anti-oxidant-related genes, NRF2, KEAP1, GCLC, GCLM and HMOX1 following 1, 8 and 24h TBH treatment compared to control samples.

ROS-mediated oxidation of DNA occurs at sites with an open chromatin structure (active genes) and this may become random in dividing cells *e.g.*, HepG2. This is reflected by pathway analysis which shows that genes containing significant 5hmC modifications are especially involved in large signaling pathways that are active during normal cellular homeostasis and DNA replication. For example, genes

involved in G-protein coupled receptor signaling, important in intracellular signal transduction, contained 5hmC modifications, as well as several histone cluster genes involved in the Wnt signaling network, which are necessary to pack newly replicated DNA [39]. Deregulated Wnt signaling is highly related to different types of cancer, including HCC [40], however, 5hmC modification in the involved genes do not result in gene expression changes. In contrast, TET binding and activity occurs at specific loci in the genome, in order to regulate DNA methylation and gene expression by inhibiting DNMT binding at these loci and converting 5mC to 5hmC by hydroxylase activity [32, 41]. However, since we also observe a loss in 5hmC adduct formation compared to untreated HepG2 cells, we do not fully exclude a role of TET1 in further oxidizing 5hmC, since its expression increases again to control levels after 24h treatment (Figure 9C). Alternatively, it is possible that DNA repair processes (*e.g.*, BER) are deleting 5hmC in specific regions of DNA.

Interestingly, an overall 5hmC gain is specifically observed in promoter and exon 1 regions, especially after 24h of TBH treatment. It is possible that these regions are more accessible during oxidant exposure and 5hmC has accumulated in these loci. However, when we integrate this data with previously obtained transcriptomic results [23], almost no differences in expression levels of genes holding 5hmC modifications following TBH treatment, are observed. In addition, an adaptive response, as previously identified (Chapter 5) to occur at the methylome level (Figure 9B) and reflected in a G2 phase arrest [23] (Figure 9A) suggesting cell normalization after an early oxidative stress-related gene expression response (Figure 9C), is not observed at the level of the hydroxymethylome. It is likely that recruitment of TET enzymes, of which the gene expression is repressed after 8h of treatment (Figure 9C), is necessary in regions containing 5hmC modifications to initiate such changes in gene expression [32, 41].

In addition, by comparing data from hMeDIP- and MeDIP-high density promoter arrays (Chapter 5), we observe that a gain in 5hmC in most cases does not lead to a loss of 5mC levels in the same loci. Moreover, in the few cases where the presence of 5hmC is accompanied by a loss of 5mC, expression levels of these genes are not differentially changed. These results conflict with the hypothesis with respect to the enzymatic activity of TET enzymes, that are also capable of preventing DNMT enzymes from binding to 5hmC enriched regions and thus of inhibiting further hypermethylation in this way and subsequently leading to increased transcriptional activity of related genes [32]. Also our results seem to demonstrate that TET proteins do not bind to 5hmC loci induced by oxidative stress.

In conclusion, this study evaluated the dynamic TBH-induced hydroxymethylome changes in combination with functional responses, DNA methylation- and gene expression changes induced by this oxidant (Figure 9). The presence of both 5mC and thymine oxidation products (5fU) in combination with the apparent lack of an indication that these 5hmC changes are regulated, substantiate the conclusion that these changes in 5hmC levels are induced by random oxidant attack and not by enzymatic regulation, for instance via TET

enzymes. Moreover, while we observe a clear toxicity-related regulation of the transcriptome and methylome in reaction to TBH-induced damage such as oxidative DNA damage (including 5fU adduct formation) and changes in cell cycle distribution (Figure 9A and B, Chapter 5), this is completely absent in the hydroxymethylome response. While gene expression and DNA methylation are regulated as a cellular response to oxidative stress 5hmC changes seem to be a random endpoint of exposure to reactive oxygen species. Overall, more research is needed to increase our understanding about the impact of chemically induced hydroxymethylation modifications on cellular toxicity and/or carcinogenicity.

Supplementary data

You can download all supplementary data using following URL:
https://mega.co.nz/#F!KoFxBB5b!gQts6mlXHKU_bwVjiGKcWQ

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CHAPTER 7

Summary and General discussion
Samenvatting en Algemene discussie

The liver is the major site for the detoxification of potentially hazardous compounds in the human body that enter through the portal vein in a concentrated form [1]. Due to its unique metabolic features, the liver is very prone to damage induced by exogenous factors, for instance resulting from the hepatic metabolism of xenobiotics, which may induce various chronic liver diseases and the development of hepatocellular carcinoma (HCC) [2]. The high health burden in particular due to increasing HCC incidence may -partly- be prevented by early detection and by improving risk assessment of potential hepatocarcinogens [3, 4].

Various types of hepatocarcinogenic compounds are considered [5]. Such compounds can be classified as either genotoxic or non-genotoxic based on mechanistic information concerning toxicity [6]. Among others, oxidative stress is hypothesized as a relevant mechanism-of-action: compounds that enter the liver, may induce oxidative stress by forming oxygen radicals directly, by increasing endogenous cellular generation of reactive oxygen species (ROS), for instance by upregulating enzymatic metabolism such as cytochrome P450 enzymes, or by decreasing antioxidant and DNA repair mechanisms. When cells are under oxidative stress, a wide range of adverse cellular effects can be induced, such as DNA damage, protein oxidation and cell cycle changes. Cells may react to this challenge by regulating cellular processes including transcriptional activation or repression. In addition, oxidative stress is also believed to alter DNA methylation patterns by interfering in active methylation and demethylation in the form of changing levels of 5-methylcytosine (5mC) and its oxidation product, 5-hydroxymethylcytosine (5hmC); respectively. This may contribute to genomic instability and thus to the induction of cancers, including HCC [7-11]. However, the underlying molecular mechanisms of oxidative stress-induced HCC development are still poorly understood [12].

This thesis is based on the hypothesis that oxidative stress-induced gene expression regulation and (epi)genetic modifications play an important role in human HCC development and promotion. For this reason, we evaluated these oxidative stress-related mechanisms on the molecular level in a human hepatoma cancer cell line, HepG2, to improve our understanding how ROS can induce whole genome gene expression and DNA (hydroxy)methylation changes that can eventually initiate and promote HCC. Since oxidative stress-related responses are probably dynamic, we considered time-series experiments important for gaining a better understanding of these cellular responses [13, 14]. Therefore, we examined gene expression alterations in combination with phenotypic endpoints in HepG2 cells after exposure to different types of oxygen radicals, focusing on correlating time-dependent changes in expression profiles. Subsequently, we investigated whether these dynamic oxidative stress-related mechanisms were HepG2 cell-specific, as well as evaluated toxicity class-specific differences (genotoxic vs non-genotoxic) related to oxidative stress.

In HCC patients, accumulation of both genetic and DNA methylation alterations can be observed and are considered to play a crucial role in changing expression of cancer-related genes in the context of the development of HCC [8]. By using a methylomics approach, we had the opportunity to integrate whole

genome DNA (hydroxy)methylation levels with whole genome gene expression. This provided us with new insights into the sequential cross-talk between genetic and active methylation/demethylation responses towards oxidative stress.

In this way, oxidative stress-specific signatures were identified which potentially improve risk assessment of new- and existing chemicals and pharmaceuticals.

Transcriptomics studies to identify specific oxidative stress-related mechanisms

In order to identify toxicologically-specific oxidative stress-related mechanisms in relation to the risk of developing HCC, we evaluated gene expression changes in combination with functional endpoints for oxidative cellular damage induced by different types of oxidative compounds as described in **Chapter 2**, **3** and **4**. For these experiments, the human hepatoma cancer cell line, HepG2, was analyzed at multiple time points in order to evaluate dynamic changes in gene expression and correlation analysis was performed to identify specific time-dependent responses towards oxidant challenge.

We therefore challenged HepG2 cells to different types of oxygen radicals, *e.g.*, superoxide formed by menadione, methyl- and hydroxyl radicals formed by TBH and hydroxyl radicals formed by H_2O_2, in increasing exposure periods (0.5, 1, 2, 4, 6, 8, 24h) as described in **Chapter 2**. Whole genome gene expression changes in combination with cell cycle changes and apoptosis measured by flow cytometry, protein oxidation by measuring carbonyl formation, and oxidative DNA damage by FPG-comet, was evaluated. In the presence of HepG2 cells, hydroxyl-, superoxide and methyl radicals were identified. This induced an increase in oxidative DNA damage and changed the cell cycle distribution which have already been associated with a high risk for HCC induction and progression [2, 15, 16].

This oxidative challenge of HepG2 cells resulted in a total of 3429 differentially expressed genes, from which 136 modified gene expressions were shared by all three oxidants. Despite these considerable differences at the gene expression level, pathway analysis showed that these genes were mostly involved in oxidative stress-related processes such as inflammatory responses, cell cycle processes and glutathione signaling. Phenotypic anchoring [17] of this molecular data demonstrated that these pathways have the strongest correlation with the measured functional endpoints for oxidative stress. Furthermore, we observed a more general acute stress response at early time points, which changed into a more compound-specific cellular response later on.

Based on results presented in this chapter, we strongly underline that time series-analysis is very useful for a better molecular understanding of oxidative stress-induced responses in cell models. Using extensive time series-analysis, we were able to identify an oxidative stress-related gene expression time cluster (BIK, AKR1C2, GCLC, GCLM, GSR, LIF, RAP1GAP, SQSTM1, GCNT3, RRAS2, SLC7A11, ASF1A, ASKR1B10, FBXO30, AGPAT9, SRXN1, PTGR1) shared

between these different types of oxidants, which contains genes involved in DNA repair [18] and glutathione metabolism, but also in HCC development [19-22]. This oxidative stress-related signature may serve as a model to recognize and identify the mode-of-action of different chemicals (drugs, potential (non-genotoxic) hepatocarcinogens) for their oxidative stress capacities (**Chapter 4**) and, therefore, may improve risk-assessment of potential carcinogenic compounds.

Whether these observed oxidative stress-related responses as discussed in **Chapter 2** are a liver cancer cell- specific phenomenon is not apparent. For that reason, we further explored these oxidative stress-related mechanisms in a cell type-specific context, as described in **Chapter 3**. To realize this objective, gene expression and cell cycle distribution data from a menadione- and H_2O_2-exposed human liver- and colon cancer cell lines (HepG2/Caco-2) were compared at multiple time points (0.5, 1, 2, 4, 8 and 24h).

The transcriptomic responses demonstrated more unique than shared significantly expressed genes compared between HepG2 and Caco-2 cells. The common differentially expressed genes in particular included oxidative stress-responsive genes such as CAT, OGG1, GCLC, HMOX1 and GSR. It seems, however, that this response was activated earlier in Caco-2 cells. Furthermore, differences in gene expression levels of important transcription factors such as NRF2, NF-κB and MYC were observed between both cell lines that thus may induce diverse downstream transcriptional and cellular events. This was confirmed by correlation analysis using cell cycle distribution data. In addition, gene expression levels of the aryl hydrocarbon receptor (AHR), an important transcription factor in toxic responses and also in non-genotoxic-induced carcinogenesis (**Chapter 4**) [23], were different between HepG2 and Caco-2 cells. Additionally, the temporal oxidative stress-related gene set, identified in **Chapter 2**, did not correlate to gene expression changes observed in Caco-2 cells, indicating that this profile is HepG2-specific. These observations explain the high number of unique DEGs observed between both cell lines.

We have thus shown that oxidative stress induces cellular damage in both cell lines, however, profound differences in the transcriptomic and cell cycle responses towards oxidative stress exist. Since these differences are of pathophysiological importance, we concluded that oxidative stress induces different transcriptional effects and outcomes in HepG2 and Caco-2 cells. In general, this work contributed to a better molecular mechanistic understanding of cell line-specific toxicity upon exposure to oxidative stress-inducing compounds.

The underlying mechanisms-of-action of such oxidative stress-inducing compounds in the liver tend to be quite complicated and are not yet fully understood. Based on their mode-of-action, these compounds can be classified as either genotoxic or non-genotoxic carcinogens [6]. Genotoxic carcinogens act by directly damaging DNA, while mechanisms involved in non-genotoxic carcinogenesis, are quite diverse (cell proliferation stimulation, apoptosis

suppression, biotransformation enzyme induction, *etc…*). Within this context it is of importance that results from previous rat studies suggest that endogenously induced oxidative stress specifically plays a key role in the mode-of-action of non-genotoxic carcinogens [24-26].

Therefore, in **Chapter 4**, the specificity of oxidative stress-related processes in chemical carcinogenesis was explored using HepG2 cells. We investigated in particular whether certain oxidative stress-related responses and mechanisms were specific for non-genotoxic carcinogenesis. For this, transcriptomic changes, DNA damage and cell cycle changes induced by different types of (non-) carcinogens that can either induce oxidative stress or not, were compared at multiple time points. For compound selection, we applied the oxidative stress-related expression profile identified in **Chapter 2**. The relevance of this oxidative stress signature for predicting oxidative stress-inducing capacities of unknown compounds was underlined by the induction of oxidative DNA damage and radical formation by Azathriopine (AZA), Tetradecanoyl-phorbol-acetate (TPA) and Diazinon (DZN) which were actually selected based on their oxidative stress-inducing properties, and the absence of oxidative DNA damage and radical formation by the selected non-oxidative stressors, Furan, Tetrachloroethylene (TCE) and D-mannitol (Dman).

We observed that oxidative stress is caused by genotoxicants, non-genotoxicants as well as non-carcinogens, and thus is not a toxicity class-specific phenomenon. An important difference observed between these oxidative stress inducers from different toxicity classes, however, was the absence of double-strand breaks formation following exposure to the non-genotoxic oxidative compound, TPA. TPA was, similar as the genotoxic, double-strand break-inducing carcinogen AZA, able to induce cell cycle changes as well as oxidative DNA damage. In addition, we observed that oxygen radical induction formed by AZA, was 10-fold higher compared to TPA. This may play a role in the significant amount of double strand breaks induced by the genotoxic carcinogen AZA and the absence of these double strand breaks following TPA exposure. Also DZN, classified as being a non-genotoxic non-carcinogen, induced single- and double strand breaks as well as several oxidative stress-related coherent transcriptional processes, as compared to the genotoxicant AZA. In addition, both compounds induced S phase arrest, whereas the non-genotoxic carcinogen TPA induces G1 phase arrest. These results indicated that DZN has the characteristics of a genotoxic carcinogen in HepG2 cells. We suggest an important role of oxidative stress in its potentially carcinogenic features.

Another interesting observation was that the selected non-genotoxic carcinogens (TPA and TCE) downregulate NRF2 expression, possibly by upregulating expression of NF-κB [27]. Also, AHR expression was increased as well as expression of cytochrome P450 genes. Both transcriptional changes can increase the formation of oxidative byproducts [28-30] and for that reason may result in an increased risk of HCC induction and progression [19-22].

All oxidative stress-inducing compounds showed a 10-fold higher number of significant gene expression changes over time compared to non-oxidative stress-inducing compounds. Approximately 50% of these genes appeared to be commonly expressed between these oxidative stress-inducing compounds and these were specifically involved in oxidative stress, DNA damage and immune responses. Moreover, these gene expression changes were regulated similarly over time. When comparing the gene expression profiles of these oxidative stress inducers to those previously found in human HCC samples [31], 362 genes could be identified which were similarly expressed in both HCC patients and in oxidant-treated HepG2 cells. These included several mitogen-activated protein kinases (MAPK) which are known to play a role in HCC by promoting cell proliferation [32]. Also genes involved in transcriptional regulation, such as histone modification, were similarly activated in both HCC patients and in oxidant-exposed HepG2 cells. This highlights that oxidative stress, induced by different types of chemicals, result in important transcriptional changes that may play a role in HCC development and promotion.

In **Chapter 4** we also observed differences in gene expression between genotoxic and non-genotoxic carcinogens that were not involved in oxidative stress or DNA damage responses, but could rather be assigned to compound-specific characteristics.

Overall, we concluded that chemically induced oxidative stress is an important risk factor in liver toxicity and carcinogenesis; however, it does not discriminate between genotoxic and non-genotoxic carcinogens.

Epigenome-based studies to further elucidate oxidative stress-related responses

It has recently been reported that oxidative stress does not only directly induce changes in gene expression, but also deregulates epigenetic processes such as DNA methylation and histone modifications. This may contribute to genomic instability and therefore the induction of carcinogenesis, including HCC development [7-11]. In carcinogenesis, DNA methylation can silence tumor suppressor genes by methylating CpG islands of their promoters, *e.g.*, prolonged ROS challenge in HCC induces methylation of the second CpG island on the E-cadherin promoter [10]. In general, it is however unclear how these free radicals are capable of impacting on the epigenome. In **Chapter 5** and **6**, we investigated the role of oxidative stress in aberrant DNA methylation and DNA hydroxymethylation modifications. In these studies, we challenged HepG2 cells with methyl- and hydroxyl radicals, formed by *tert*-butyl hydroperoxide (TBH), in order to investigate their effect on DNA (hydroxy)methylation and their interference with the interplay between the transcriptome and methylome.

In **Chapter 5**, we first investigated the impact of methyl radicals on 5mC levels, since direct binding of these radicals to deoxycytidine in a cell-free system has been observed previously [33]. In addition, we assessed the interplay between the transcriptome and methylome in response to methyl and hydroxyl radical

challenge induced by TBH at three different time points (1, 8 and 24h) by applying whole genome methylation and gene expression analysis. By combining these micro-array-based techniques with ESR spectroscopy for free radical detection and LC-MS/MS to quantify 5mC levels, we concluded that methyl radicals were not able to function as direct methyl donors for DNA methylation in HepG2 cells.

The observed DNA methylation changes rather suggested an adaptive response towards the oxidative stress-induced gene expression responses. In more detail, the transcriptome initially reacted in an oxidative and alkylating stress-related response to TBH which was followed by a gene expression response that is associated with cell survival after 24h of treatment. For example, an initially increased expression of the transcription factor NRF2 and further downstream genes involved in the antioxidant machinery, was observed following 8h of TBH challenge and returned to control expression levels after 24h. Results showed that the majority of gene expression changes induced by 8h of TBH treatment were transient. We suggest that this initial gene expression response towards the hydroxyl- and methyl radical challenge is counter-regulated by the methylome. In contrast, it has been previously documented that ROS can directly induce DNA methylation. For example, reduced catalase levels in HCC are believed to be caused by ROS-induced hypermethylation of the catalase promoter [34]. Our results show a decrease in catalase expression following ROS challenge. However, no increase in 5mC levels was observed during oxidative stress in HepG2 cells, suggesting that other mechanisms control catalase expression in HepG2 cells.

Interestingly, genes transiently induced after oxidative- and alkylating stress such as MAPK14, as well as other responsive genes, were found to be upregulated in liver samples from HCC patients [31]. Since such genes were hypermethylated in promoter or exon 1 regions following 24h TBH treatment, this might indicate that TBH initially induces a carcinogenic phenotype after 8h exposure which is opposed by the methylome after 24h to regain normal cell function.

This process of DNA methylation is dynamically regulated by active methylation and demethylation and plays a crucial role in biological processes and hepatocarcinogenesis [7, 35-37]. In other studies; the Ten-Eleven Translocation (TET) proteins have been found to oxidize 5mC to form 5hmC [38], however, it is not clear how active demethylation is regulated by oxidative stress-related processes. This was further explored in **Chapter 6**, where we hypothesized that free oxygen radicals induced by TBH, are able to generate 5hmC directly.

In this study, we compared whole genome hydroxymethylation, DNA methylation and gene expression analysis. These microarray-based technologies were combined with the identification of different oxidation products of cytosine and thymine in HepG2 cells exposed to hydroxyl- and methyl radicals, formed by TBH, at multiple time points (1h, 8h and 24h). Using LC-MS/MS, we found that TBH significantly increased levels of 5hmC and oxidized thymine. In depth analysis of hydroxymethylated regions, showed us that these 5hmC modifications were highly dynamic and increased with prolonged TBH exposure. Also more hyper-5hmC

promoter regions compared to hypo-5hmC promoter regions were observed, which indicated that there was a detectable gain in 5hmC modifications in these specific regions. Surprisingly, these promoter-specific 5hmC modifications had only limited effects on gene expression. Additionally, also an insignificant overlap of 5hmC changes with active demethylation was observed. Presumably, the recruitment of TET proteins is necessary to initiate changes in gene expression due to hydroxymethylation [39, 40]. For these reasons, we suggest that the observed 5hmC modifications were mainly randomly induced by TBH and did not seem to be regulated.

General discussion and conclusions

In general, our hypothesis that oxidative stress-induced gene expression regulation and (epi)genetic modifications play an important role in human HCC development and promotion was partly confirmed. In particular, we have improved our molecular understanding by correlating functional endpoints of oxidative stress to responses of the transcriptome and showed especially the induction of pathways involved in cell cycle changes, DNA damage and glutathione signaling. These functional endpoints of oxidative stress that we observed in oxidant-exposed HepG2 cells, such as the presence of oxidative lesions in DNA, are associated with a higher risk for HCC development in humans as previously described [15, 16]. Moreover, activation of transcription factors such as NRF2, NF-κB, FOS, TGFβ is related to HCC [41-44] which in our study were upregulated by different types of oxidants and carcinogens. TGFβ can also promote tumor cell invasion by inducing epithelial-mesenchymal transition (EMT) [45] which we observed to be a HepG2-specific oxidative stress-induced pathway.

Furthermore, the detected oxidative DNA damage may induce single-strand breaks which can accumulate in cells over time and evolve into double-strand breaks [46] leading to chromosomal aberrations, translocations and subsequent genomic instability. The formation of such double strand breaks was observed in HepG2 cells challenged with compounds inducing high radical formation and oxidative DNA damage (AZA and DZN).

Overall, we report a range of results which confirm the hypothesis that induction of oxidative stress is associated with the onset of HCC.

We observed, however, that ROS formation is not limited to either genotoxic or non-genotoxic challenge in HepG2 cells, as also observed in a recent toxicogenomics study in mice [47]. We suggest, however, that the difference in mode-of-action between oxidative stress-inducing genotoxic carcinogens and non-genotoxic carcinogens relates to how oxidative stress is induced and to what extent; *e.g.*, exogenous ROS formation by metabolism of genotoxicants versus indirect and endogenous formation of ROS by cytochrome p450 activation, AhR binding, PKC activation and GSH depletion [48, 49] induced by non-genotoxic carcinogens.

While a high number of significant changes in the transcriptome in response towards oxidative stress was induced and strongly correlated to toxicity

and carcinogenic processes, our DNA methylation and hydroxymethylation studies did not provide us with evidence that these epigenetic processes contribute to this carcinogenic profile. In other studies, accumulation of both 5mC and 5hmC modifications, however, have been linked to HCC, and was considered to play a role in the initial alteration of cancer-related gene expressions in HCC development [36]. In contrast, in our studies, the high number of changes in DNA methylation was observed to be rather an adaptive response towards the initial oxidative stress-related response of the transcriptome. Since methyltransferase genes were not differentially expressed during oxidant challenge, we suggest that this adaptive response of the methylome occurred independently from the expression of different DNMT enzymes. Such a DNMT-independent adaptive response was observed previously in the rat brain following acute restraint stress [50] as well as in a human B lymphoblast cell line exposed to low dose ionizing radiation [51]. Modifications in the hydroxymethylome, on the other hand, did not result in such adaptive response towards the transcriptome, neither in carcinogenic-related responses. These changes in 5hmC levels had only limited effects on the transcriptome.

Possibly, in the human *in vivo* situation, toxic effects which may lead to HCC development and progression are only induced at the level of the epigenome following higher and/or more prolonged doses of ROS.

Lastly, we found that functional and transcriptomic responses in HepG2 cells differ from those in Caco-2 cells that were under oxidative stress. When extrapolating these results to the physiological conditions in humans, we suggest that while a particular compound with oxidative stress-inducing capacities is capable of inflicting damage in multiple target organs in the human body, the decisive factor being how this compound is taken up and transferred throughout the human system, this may still result in different tissue-specific responses.

In conclusion, results discussed in this thesis provided us with new molecular insights in temporal oxidative stress-induced responses in the transcriptome which correlated to HCC development and how these differ between cell types. Changes induced in the epigenome did, however, not contribute to this carcinogenic profile but we demonstrated an important role of the methylome in counter-regulating earlier oxidative stress responses of the transcriptome in order to restore normal cellular function. By using excessive time series-analyses, we were able to identify a HepG2-specific oxidative stress-related gene expression profile that may contribute to the improvement of hazard identification and mechanistic knowledge of toxicological responses specifically in the liver induced by different types of oxidative stress-inducing (non-)hepatocarcinogens. In contrast to what is shown in previous studies in rat livers [24-26], the use of this human liver cell line in a comparative study with different types of (non-)hepatocarcinogens, showed us that oxidative stress is not a non-genotoxic carcinogen-specific phenomenon and should therefore not be used to discriminate genotoxic from non-genotoxic carcinogens. Finally, while we observed a clear correlation and

regulation between functional endpoints, the transcriptome and the methylome in response to oxidative stress, induced changes in the hydroxymethylome seemed to be a result of random processes.

Altogether, these results contribute towards the unraveling of unknown oxidative stress-related mechanisms in the development of HCC. In addition, this work lays the foundation for further improvement of risk assessment of possible hepatocarcinogens. This may thus create new opportunities for advancing preventive measures and early treatment of HCC.

Future recommendations

New mechanistic information concerning oxidative stress-related responses, induced by different types of oxidative compounds, has been obtained by using HepG2 cells. Also a HepG2-specific oxidative stress-related expression profile was identified and applied for better recognition and mechanistic understanding of oxidative stress induced cell damage by different types of hepatocarcinogens. It is important to stress, however, that only a limited number of compounds with only a single dose of each chemical in only one *in vitro* cell model, were studied in this thesis. For this reason, additional validation efforts need to be performed using more hepatocarcinogens and non-hepatotoxic compounds followed by validation in different cell models.

Since HepG2 cells have multiple limitations, such as a low expression of important metabolic and liver toxicity-related genes and an aberrant karyotype compared to normal liver cells, validation efforts in other *in vitro* liver models are of interest. Primary Human Hepatocytes (PHH) are considered to represent the best *in vitro* cell system for liver studies [52]. The use of these cells in toxicity screening, however, does also encounter problems due to difficulties in culturing procedures and inter-individual differences between liver donors. Additionally, due to limited availability of fresh human liver samples, these PHH are expensive which hinder the use of PHH for large screening purposes [53]. Recently, three dimensional, organotypical cell cultures, such as liver spheroids or organoids [54], have been developed. In spheroids, co-cultures of PHH or liver cancer cell lines in combination with kupffer cells are mostly used to form a micro-tissue while in organoids, stem cells are derived from a human tissue of interest [54]. In both models, cells are allowed to self-organize in the matrix in a way that resembles the *in vivo* situation [54, 55]. In general, compared to the 2D culture of cell monolayers, these 3D models more closely mimic native tissues since the cellular microenvironment established in the 3D models often plays a significant role in disease progression and cellular responses to drugs [56, 57]. Together with pluripotent stem cells, which can be transformed to fully metabolic competent human hepatocytes [58], these new *in vitro* models are promising in human toxicity screening [59].

Next to the implementation of these new *in vitro* cell models, it is important to connect also this data from such new *in vitro* studies with existing data from clinical studies in HCC patients, as we have done in our studies using HepG2 cells

for predicting to what extent oxidative stress-induced mechanisms can contribute to the development of HCC [31].

Our epigenome-based studies provided us with relevant and innovative information about adaptive responses of the methylome in response to transcriptomic oxidative stress responses. First, because DNA methylation changes did only partly correlate to gene expression changes, also other cellular processes *e.g.*, microRNAs, protein phosphorylation, and acetylation or methylation of histones, should be investigated since these may act in concert with DNA methylation to maintain normal gene expression in response to oxidative and alkylating stress. In order to obtain a better understanding of all these epigenetic processes and their responses to oxidative stress, additional experiments have to be performed and correlated to gene expression changes. For example, inactivation of the oxidative stress-related transcription factor NRF2 using interference RNA (siRNA or shRNA), may provide us with additional information concerning our hypothesis on the disturbance of the interplay between the transcriptome and epigenome - and even the loss of this - in response to oxidative stress. In addition, our microarray experiments are restricted to specific annotated regions, while next generation sequencing (NGS) offers additional information in terms of near-base pair data, splicing variants, non-coding RNA or novel transcripts with a higher specificity and sensitivity. By using this advanced 'omics technique we may be able to identify specific regions in the genome that are possibly more susceptible to oxidative damage and translate this data into relevant biological information at multiple time points. Also more effort is needed to increase our knowledge about oxidative stress-induced 5hmC modifications. For example, TET1 inactivation in an oxidative stress challenged *in vitro* liver cell model can be established using siRNA or shRNA approaches to evaluate its role in oxidative stress-induced 5hmC modifications [60].

In general, the rapid development and further improvement of these new techniques can further elucidate molecular mechanisms of chemically induced oxidative stress in the onset of HCC.

De lever is het belangrijkste orgaan voor het ontgiften van potentieel gevaarlijke stoffen die via de poortader in een geconcentreerde vorm in het menselijk lichaam opgenomen worden [1]. Door zijn unieke metabole functies, is de lever erg gevoelig voor schade geïnduceerd door exogene stoffen. De omzetting van bepaalde stoffen naar actieve metabolieten is bijvoorbeeld gerelateerd aan chronische leverziekten en hepatocellulair carcinoom (HCC) [2]. De consequenties van het ziektebeeld als gevolg van toenemende HCC incidentie in het bijzonder kan - gedeeltelijk - worden voorkomen door vroege opsporing en door het verbeteren van de risicobepaling van potentiële levercarcinogenen [3, 4].

Verschillende soorten levercarcinogenen zijn gedefinieerd [5]. Dergelijke types kunnen geclassificeerd worden als wel- of niet-genotoxisch, gebaseerd op mechanistische informatie over toxiciteit [6]. Oxidatieve stress wordt geacht een relevant werkingsmechanisme hierin te zijn. Stoffen die de lever binnen komen kunnen oxidatieve stress induceren door het rechtstreeks vormen van zuurstofradicalen door de intracellulaire productie van reactieve zuurstof species (ROS) te stimuleren. Dit door bijvoorbeeld het enzymatische metabolisme van cytochroom P450-enzymen te verhogen, of door verlaging van antioxidant- en DNA herstelmechanismen. Wanneer cellen onder oxidatieve stress staan, kunnen diverse ongewenste cellulaire effecten worden geïnduceerd, zoals DNA-beschadiging, eiwit oxidatie en celcyclus veranderingen. Cellen kunnen reageren op deze schade door het reguleren van cellulaire processen, waaronder transcriptionele activatie of repressie. Bovendien wordt ook verondersteld dat oxidatieve stress DNA methylatie patronen kan veranderen door te interfereren met inactieve methylering en demethylering, respectievelijk in de vorm van veranderende niveaus van 5-methylcytosine (5MC) en het oxidatieproduct, 5-hydroxymethylcytosine (5hmC). Dit kan bijdragen tot genetische instabiliteit en dus de vorming van kanker, inclusief HCC [7-11]. Echter, de onderliggende moleculaire mechanismen van oxidatieve stress-geïnduceerde HCC ontwikkeling zijn nog steeds niet helder [12].

Dit proefschrift is gebaseerd op de hypothese dat oxidatieve stress-geïnduceerde genexpressie regulatie en (epi-)genetische modificaties een belangrijke rol spelen bij humane HCC ontwikkeling en verdere progressie. Daarom werden in een humane leverkanker cellijn, HepG2, deze oxidatieve stress gerelateerde mechanismen op moleculair niveau onderzocht om onze kennis te verbeteren hoe ROS genexpressie en DNA (hydroxy)methylatie over het gehele genoom kan veranderen wat uiteindelijk kan leiden tot HCC ontwikkeling en progressie. Omdat deze oxidatieve stress reacties waarschijnlijk dynamisch zijn, hebben we gebruik gemaakt van meerdere tijdreeks-experimenten voor een beter begrip van deze cellulaire reacties over de tijd [13, 14]. Op deze manier onderzochten we genexpressie veranderingen in combinatie met fenotypische eindpunten in HepG2 cellen na blootstelling aan verschillende zuurstofradicalen, gericht op het correleren van tijdsafhankelijke veranderingen in genexpressieprofielen. Vervolgens hebben we onderzocht of deze dynamische oxidatieve stress mechanismen HepG2-specifiek waren, alsook de specifieke

oxidatieve stress-gerelateerde verschillen in de geëvalueerde toxiciteits klassen (genotoxische vs niet-genotoxisch).

Accumulatie van zowel genetische- en DNA methylatie veranderingen worden geobserveerd bij HCC patiënten en blijken een cruciale rol te spelen bij een veranderde expressie van kanker-gerelateerde genen in het kader van de ontwikkeling van HCC [8]. Door het gebruik van een epigenetische benadering, bestaat de mogelijkheid om DNA (hydroxy)methylatie niveaus te integreren met genexpressie veranderingen over het gehele genoom. Dit gaf ons nieuwe inzichten in de verbinding tussen genetische- en actieve (de)methylatie reacties op oxidatieve stress.

Op deze manier werden oxidatieve stress-specifieke genen geïdentificeerd die mogelijk de risicobepaling van nieuwe- en bestaande chemische en farmaceutische producten kunnen verbeteren.

Transcriptomics studies om specifieke oxidatieve stress-gerelateerde mechanismen te identificeren

Om toxicologisch-specifieke oxidatieve stress-gerelateerde mechanismen met betrekking tot het risico op HCC te identificeren, hebben we genexpressie veranderingen geëvalueerd in combinatie met functionele eindpunten voor oxidatieve cellulaire schade, geïnduceerd door verschillende oxidatieve stoffen zoals beschreven in **Hoofdstuk 2**, **3** en **4**. Voor deze experimenten, hebben we de humane lever kankercellijn, HepG2, geanalyseerd opverschillende tijdstippen om zo dynamische veranderingen in genexpressie te evalueren en vervolgens iseen correlatieanalyse uitgevoerd om specifieke tijdsafhankelijke reacties te identificeren.

Daarom hebben we HepG2 cellen blootgesteld aan verschillende zuurstofradicalen, zoals superoxide gevormd door menadione, methyl- en hydroxylradicalen gevormd door TBH en hydroxylradicalen gevormd door waterstofperoxide (H_2O_2) in toenemende perioden van blootstelling (0,5, 1, 2, 4, 6, 8, 24u) zoals beschreven in **Hoofdstuk 2**. Genexpressie veranderingen over het gehele genoom in combinatie met celcyclus veranderingen en apoptose zijn gemeten met flow cytometrie, eiwit oxidatie aan de hand van de bepaling van carbonyl-niveaus en oxidatieve DNA schade met de FPG comet. Met behulp van electrospin resonantie (ESR) spectrometrie werden in aanwezigheid van HepG2 cellen hydroxyl-, superoxide en methylradicalen geïdentificeerd. De blootstelling aan oxidanten veroorzaakte een toename van oxidatieve DNA schade en veranderde de celcyclusverdeling die eerder al gerelateerdwas met een verhoogd risico op inductie van HCC en verdere progressie [2, 15, 16].

Deze oxidatieve blootstelling van HepG2-cellen resulteerde in een totaal van 3429 differentieel tot expressie komende genen, waarvan 136 gemodificeerde genexpressies door alle drie de oxidanten. Ondanks deze aanzienlijke verschillen op het niveau van genexpressie, toonde de pathway analyse ons dat deze genen meestal betrokken zijn bij oxidatieve stress processen zoals ontstekingsreacties, celcyclus processen en glutathion signalering. Door middel van fenotypische verankering [17] tussen deze moleculaire gegevens is aangetoond dat deze

pathways de sterkste correlatie hadden met de gemeten functionele eindpunten voor oxidatieve stress. Daarnaast zagen we een algemene acute stressreactie op vroege tijdstippen, die later in een meer oxidant-specifieke cellulaire respons veranderde.

Op basis van de resultaten in dit hoofdstuk willen we nog eens benadrukken dat tijdreeks-analyses erg nuttig zijn voor een beter moleculair begrip van oxidatieve stress-geïnduceerde reacties in cel modellen. Met behulp van een uitgebreide tijdreeks-analyse waren we in staat om een oxidatieve stress-gerelateerde genexpressie tijd cluster te identificeren (BIK, AKR1C2, GCLC, GCLM, GSR, LIF, RAP1GAP, SQSTM1, GCNT3, RRAS2, SLC7A11, ASF1A, ASKR1B10, FBXO30, AGPAT9, SRXN1, PTGR1) met genen betrokken in DNA-herstel [18] en glutathione metabolisme, maar ook in de ontwikkeling van HCC [19-22]. Dit oxidatieve stress signatuur kan als model dienen om de werking van verschillende chemische stoffen (drugs, mogelijke (niet-genotoxisch) hepatocarcinogenen) te herkennen en te identificeren op hun oxidatieve stress capaciteiten (**Hoofdstuk 4**) en daarom ook om de risico-evaluatie van mogelijke kankerverwekkende stoffen te verbeteren.

Of deze waargenomen oxidatieve stress-gerelateerde reacties, zoals besproken in **Hoofdstuk 2,** een leverkanker cel-specifiek fenomeen zijn is niet duidelijk. Om die reden hebben we deze oxidatieve stress-gerelateerde mechanismen in een celtype-specifieke context nader onderzocht zoals beschreven in **Hoofdstuk 3**. Om deze doelstelling te realiseren, hebben we de gegevens van genexpressie en celcyclus verdeling aan een menadione- en H_2O_2-blootgestelde humane lever- en colonkanker cellijn (HepG2 / Caco-2) met elkaar vergeleken gedurende verschillende tijdstippen (0.5, 1, 2, 4, 8 en 24u).

De reactie van het transcriptoom bestond uit aanzienlijk meer unieke dan overeenkomstige differentieel tot expressie komende genen tussen HepG2- en Caco-2 cellen. Deze overeenkomstige genen bestonden vooral uit oxidatieve stress-responsieve genen zoals CAT, OGG1, GCLC, HMOX1 en GSR. Het lijkt er echter op dat deze reactie in Caco-2 cellen eerder werd geactiveerd. Bovendien werden verschillen op genexpressie niveaus van belangrijke transcriptiefactoren zoals NRF2, NF-κB en MYC waargenomen tussen beide cellijnen die elk diverse downstream transcriptionele en cellulaire veranderingen kunnen induceren. Dit werd bevestigd door correlatieanalyse met gegevens over veranderingen in de celcyclus. Bovendien was het genexpressie niveau van de aryl hydrocarbon receptor (AHR), een belangrijke transcriptiefactor in toxische reacties en ook in niet-genotoxische geïnduceerde carcinogenese (**Hoofdstuk 4**) [23], verschillend tussen HepG2 en Caco-2 cellen. Bijkomend, de oxidatieve stress-gerelateerde genen-set, die in **Hoofdstuk 2** werd geïdentificeerd, correleerde niet met genexpressie veranderingen waargenomen in Caco-2 cellen, wat aangeeft dat dit profiel HepG2-specifiek is. Deze waarnemingen verklaren het hoge aantal unieke differentieel tot expressie komende genen zoals waargenomen tussen beide cellijnen.

We hebben dus aangetoond dat oxidatieve stress cellulaire schade induceert in beide cellijnen maar dat er aanzienlijke verschillen in het transcriptoom en celcyclus verdeling ontstaan. Omdat deze verschillen van pathofysiologisch belang zijn, hebben we geconcludeerd dat oxidatieve stress verschillende transcriptionele effecten en eindresultaten in HepG2- en Caco-2 cellen induceert. Over het algemeen heeft dit alles ertoe geleid in een beter moleculaire mechanistische in cellijn-specifieke toxiciteit na blootstelling aan oxidatieve stress-inducerende stoffen.

De onderliggende werkingsmechanismen van dergelijke oxidatieve stress-inducerende stoffen in de lever zijn ingewikkeld en worden nog niet volledig begrepen. Op basis van hun werking, kunnen deze stoffen geclassificeerd worden als wel- of niet-genotoxische carcinogenen [6]. Genotoxische carcinogenen induceren rechtstreeks schade aan het DNA, terwijl heel diverse mechanismen betrokken zijn bij niet-genotoxische carcinogenese (cel proliferatie stimulatie, onderdrukking van apoptose, biotransformatie, enzyminductie, etc…). De resultaten van eerdere studies in de rat suggereren dat endogeen gevormde oxidatieve stress een sleutelrol speelt in het werkingsprincipe van deze niet-genotoxische carcinogenen [24-26].

Daarom is in **Hoofdstuk 4**, de specificiteit van oxidatieve stress-gerelateerde processen in de chemische carcinogenese verder onderzocht met behulp van HepG2 cellen. We hebben in het bijzonder gekeken of bepaalde oxidatieve stress-gerelateerde reacties en mechanismen specifiek waren voor niet-genotoxische carcinogenese. Hiervoor werden transcriptoom veranderingen, DNA schade en celcyclus veranderingen bij verschillende (niet-) carcinogenen die al dan niet oxidatieve stress kunnen induceren vergeleken op verschillende tijdstippen. Voor de selectie van stoffen pasten we het oxidatieve stress genexpressieprofiel toe dat werd geïdentificeerd in **Hoofdstuk 2**. De relevantie van deze oxidatieve stress signatuur voor het voorspellen van oxidatieve stress-inducerende capaciteiten van onbekende stoffen werd verder benadrukt door de inductie van oxidatieve DNA schade en radicalen door Azathriopine (AZA), Tetradecanoyl-Forbol-Acetaat (TPA) en Diazinon (DZN), die daadwerkelijk geselecteerd werden op basis van hun oxidatieve stress-inducerende eigenschappen. Daarnaast bleek de afwezigheid van oxidatieve DNA schade en radicalen door de geselecteerde niet-oxidatieve stressoren, Furan, tetrachloorethyleen (TCE) en D-mannitol (Dman).

We zagen dat oxidatieve stress werd veroorzaakt door genotoxische, niet-genotoxische, als ook niet-carcinogenen en is daarom geen toxiciteit klasse-specifiek fenomeen. Een belangrijk verschil tussen deze oxidatieve stress-inducerende stoffen van verschillende toxiciteit-klassen was echter de afwezigheid van dubbelstrengs DNA breuken na blootstelling aan niet-genotoxische oxidatieve stof, TPA. TPA was, zoals de genotoxische dubbelstrengs DNA breuk-inducerende carcinogeen AZA, in staat om veranderingen in de celcyclus en oxidatieve DNA schade te induceren. Daarnaast namen we waar dat de vorming van zuurstofradicalen door AZA 10 keer hoger was vergeleken met TPA. Dit kan een rol spelen in de aanzienlijke hoeveelheid dubbelstrengs DNA breuken veroorzaakt

door de genotoxische kankerverwekkende stof AZA en de afwezigheid van deze dubbele breuken na TPA blootstelling. Ook DZN, geclassificeerd als zijnde een niet-genotoxische, niet-carcinogeen, induceerde enkel- en dubbelstrengs DNA breuken evenals gelijkaardige oxidatieve stress-gerelateerde coherente transcriptionele processen, in vergelijking met de genotoxische stof AZA. Daarnaast induceerden beide stoffen S fase arrest, terwijl de niet-genotoxisch carcinogeen, TPA, arrest in de G1 fase veroorzaakte. Deze resultaten geven aan dat DZN de kenmerken heeft van een genotoxisch kankerverwekkende stof in HepG2 cellen. Verder suggereren we een belangrijke rol van oxidatieve stress in zijn potentiele kankerverwekkende eigenschappen.

Een andere interessante bevinding was dat de geselecteerde niet-genotoxische carcinogenen (TPA en TCE) NRF2 expressie doet dalen, mogelijk door verhoogde expressie van NF-κB [27]. Ook nam de expressie van AHR toe, evenals de expressie van cytochroom P450 genen. Beide transcriptionele veranderingen kan de vorming van oxidatieve bijproducten [28-30] verhogen en kunnen daarom leiden tot een verhoogd risico op HCC inductie en verdere progressie [19-22].

Alle oxidatieve stress inducerende stoffen vertoonden een 10-maal hoger aantal significante veranderingen in genexpressie over de tijd in vergelijking met niet-oxidatieve stress inducerende stoffen. Ongeveer 50% van deze genen bleken dezelfde expressie-niveaus te hebben tussen deze oxidatieve stress inducerende verbindingen en waren specifiek betrokken bij oxidatieve stress, DNA schade en immuunreacties. Bovendien werden deze genexpressie veranderingen hetzelfde gereguleerd over de tijd. Wanneer we de genexpressieprofielen geïnduceerd door deze oxidatieve stress inductoren vergelijken met eerder gemeten expressieprofielen van humaan HCC monsters [31], konden we 362 genen identificeren die in zowel HCC patiënten en oxidant behandelde HepG2 cellen vergelijkbaar tot expressie kwamen. Deze omvatten verscheidene mitogeen geactiveerde eiwitkinasen (MAPK) waarvan bekend is dat deze een rol spelen in het bevorderen van de celdeling bij HCC [32]. Ook genen betrokken bij transcriptionele regulatie, zoals histonmodificatie genen, werden eveneens geactiveerd in zowel HCC patiënten als in de aan oxidant blootgestelde HepG2 cellen. Dit wijst erop dat oxidatieve stress, geïnduceerd door verschillende soorten chemicaliën, kan leiden tot belangrijke transcriptionele veranderingen die een rol spelen bij HCC ontwikkeling en verdere progressie.

In **Hoofdstuk 4** zagen we ook verschillen in genexpressie tussen genotoxische en niet-genotoxische carcinogenen die niet betrokken waren bij oxidatieve stress of reacties op DNA-schade, maar die eerder konden worden toegewezen aan stof-specifieke kenmerken.

Kortom, we concluderen dat chemisch-geïnduceerde oxidatieve stress een belangrijk risicofactor is voor levertoxiciteit en –kanker, maar niet het onderscheid maakt tussen genotoxische en niet-genotoxische carcinogenen.

Epigenoom gebaseerde studies om oxidatieve stress-gerelateerde reacties te verhelderen

Het is recent gerapporteerd dat oxidatieve stress niet alleen rechtstreeks veranderingen in genexpressie induceert, maar ook epigenetische processen kan ontregelen zoals DNA methylering en histonmodificaties. Dit kan bijdragen tot genomische instabiliteit en dus de ontwikkeling van kanker, zoals HCC [7-11]. Tijdens de carcinogenese kan DNA methylatie tumorsuppressorgenen uitschakelen door het methyleren van CpG eilanden in hun promotor regio. Langdurige ROS blootstelling in HCC patiënten induceert bijvoorbeeld methylering van het tweede CpG eiland op de E-cadherine-promotor [10]. Over het algemeen is het echter onduidelijk hoe deze vrije radicalen invloed hebben op het epigenoom. In **Hoofdstuk 5** en **6**, onderzochten we de rol van oxidatieve stress in afwijkende DNA-methylatie en DNA hydroxymethylatie. In deze studies stelden we HepG2 cellen bloot aan methyl- en hydroxyl radicalen, gevormd door tert-butylhydroperoxide (TBH), om zo hun effect op DNA (hydroxy)methylering en de verstoring van de interactie tussen het transcriptoom en methyloom te onderzoeken.

In **Hoofdstuk 5**, onderzochten we eerst de invloed van methyl radicalen op 5mC niveaus, omdat directe binding van deze radicalen op deoxycytidine in een cel vrij systeem eerder waargenomen is [33]. Bovendien hebben we de wisselwerking tussen het transcriptoom en methyloom als reactie op methyl en hydroxyl radicaal blootstelling op drie verschillende tijdstippen (1, 8 en 24u) geanalyseerd door het toepassen van methylatie en genexpressie analyse over het gehele genoom. Door deze micro-array gebaseerde technieken te combineren met ESR spectroscopie de detectie van vrije radicalen , en LC-MS/MS voor het kwantificeren van 5mC niveaus, concludeerden we dat in HepG2 cellen methylgroepen niet kunnen functioneren als directe methyldonoren voor DNA methylatie.

De waargenomen DNA methylatie veranderingen suggereerden eerder een adaptieve respons op de oxidatieve stress geïnduceerde genexpressie reacties. Om precies te zijn, het transcriptoom reageerde aanvankelijk met een oxidatieve en alkylerende stress respons op TBH gevolgd door een genexpressie respons die geassocieerd is met overleving van cellen 24 uur na de behandeling. Zo werd een verhoogde expressie van de transcriptiefactor NRF2 en verdere downstream genen betrokken bij de antioxidant reactie waargenomen na 8u TBH blootstelling gevolgd door een verlaging van deze expressieniveaus terug naar controlelevels op 24u. De resultaten toonden aan dat de meerderheid van de genexpressie veranderingen bij 8u TBH behandeling tijdelijk waren. We vermoeden dat deze vroege genexpressie reactie op de hydroxyl- en methylradicalen blootstelling contra-gereguleerd wordt door het methyloom. Deze resultaten spreken de eerder beschreven bevindingen dat ROS rechtstreeks DNA-methylatie kan induceren tegen. Zo worden gereduceerde catalase niveaus in HCC verondersteld te worden veroorzaakt door ROS-geïnduceerde hypermethylering van de catalase promotor [34]. Onze resultaten laten ook een afname in catalase expressie zien na blootstelling aan ROS. Echter, we namen geen verhoging in 5mC niveaus waar in de catalase promotor van HepG2-

cellen na oxidatieve stress, wat suggereert dat andere mechanismen de catalase expressie bepaalt in HepG2 cellen.

Een interessante bevinding was dat genen die tijdelijk geïnduceerd werden na oxidatieve- en alkylerende stress zoals MAPK14, evenals andere responsieve genen, ook een verhoogde expressie vertonen in leverweefsel van HCC-patiënten [31]. Aangezien dergelijke genen gehypermethyleerd werden in het exon 1-gebied van de promotor regio na 24u TBH blootstelling, kan dit erop wijzen dat TBH aanvankelijk een kankerverwekkend fenotype induceert na 8h blootstelling, die vervolgens wordt tegengewerkt door het methyloom na 24 uur om opnieuw een normale cel functie te verkrijgen.

Dit proces van DNA methylatie wordt dynamisch gereguleerd door actieve (de)methylering en speelt een cruciale rol in biologische processen en levercarcinogenese [7, 35-37]. In voorgaande studies werd ontdekt dat de 'Ten Eleven Translocatie (TET) enzymen 5mC oxideren om zo 5hmC te vormen [38], enkel is het niet duidelijk hoe actieve demethylering wordt geregeld door oxidatieve stress-gerelateerde processen. Dit werd verder onderzocht in **Hoofdstuk 6**, waar we hypothetiseren dat de vrije zuurstofradicalen gevormd door TBH in staat zijn om 5hmC rechtstreeks te genereren.

In deze studie hebben we hydroxymethylering over het gehele genoom vergeleken met DNA methylatie en genexpressie. Deze op microarray gebaseerde technologieën werden gecombineerd met de identificatie van verschillende oxidatieproducten van cytosine en thymine in - aan hydroxyl en methylradicalen blootgestelde - HepG2 cellen gedurende verschillende tijdstippen (1, 8 en 24u). Met gebruik van LC-MS/MS, vonden we dat TBH de niveaus van 5hmC en geoxideerd thymine significant liet stijgen. Verdere analyse van gehydroxymethyleerde regio's, liet ons vervolgens zien dat deze 5hmC wijzigingen zeer dynamisch waren en toenamen met langdurige blootstelling aan TBH. Er werden ook meer hyper-5hmC promotorgebieden waargenomen in vergelijking met hypo-5hmC promotorgebieden, waaruit bleek dat er een detecteerbare toename in 5hmC modificaties was in deze specifieke gebieden. Verrassenderwijs hadden deze promotor-specifieke 5hmC modificaties slechts beperkte effecten op de genexpressie. Daarnaast werd er een verwaarloosbare overlap van 5hmC veranderingen in vergelijking met actieve demethylering waargenomen. Vermoedelijk is de werving van TET enzymen nodig om veranderingen in genexpressie te initiëren door hydroxymethylering [39, 40]. Om deze redenen veronderstellen we ook dat de waargenomen 5hmC wijzigingen voornamelijk willekeurig werden geïnduceerd door TBH en niet gereguleerd worden.

Algemene discussie en conclusie

In het algemeen werd onze hypothese dat oxidatieve stress-geïnduceerde genexpressie regulatie en epigenetische modificaties een belangrijke rol spelen bij humane HCC ontwikkeling en progressie gedeeltelijk bevestigd. In het bijzonder is ons moleculair begrip verbeterd door het correleren van functionele eindpunten

van oxidatieve stress aan veranderingen van het transcriptoom, die vooral betrokken zijn in de inductie van pathways betrokken bij celcyclus veranderingen, DNA schade en glutathion signalering. Deze functionele eindpunten van oxidatieve stress, zoals de aanwezigheid van oxidatieve laesies in het DNA, die we waargenomen hebben in - aan oxidant - blootgestelde HepG2-cellen, zijn geassocieerd met een verhoogd risico op HCC ontwikkeling zoals eerder bij mensen beschreven werd [15, 16]. Bovendien wordt de activatie van transcriptiefactoren zoals NRF2, NF-κB, FOS en TGFβ in relatie gebracht met HCC [41-44]. De expressie van deze transciptiefactoren werd in ons onderzoek verhoogd door verschillende soorten oxidanten en carcinogenen. De verhoogde activatie van TGFβ kan op zijn beurt weer leiden tot metastase door het induceren van epitheliale-mesenchymale transitie (EMT) [45], die we zelf waargenomen hebben als een HepG2-specifieke oxidatieve stress geïnduceerd pathway.

Bovendien kan de gedetecteerde oxidatieve DNA schade enkelstrengs DNA breuken veroorzaken die kunnen ophopen in cellen over de tijd en kunnen leiden tot het ontstaan van dubbelstrengs DNA breuken [46] wat leidt tot afwijkingen, translocaties en daaropvolgende genomische chromosomale instabiliteit. De vorming van dergelijke dubbelstrengs breuken werd waargenomen in HepG2 cellen blootgesteld aan stoffen die grote hoeveelheden zuurstofradicalen vormen en oxidatieve DNA-schade induceren (AZA en DZN).

Kortom, we rapporteren een reeks van resultaten die de hypothese dat inductie van oxidatieve stress geassocieerd is met het ontstaan van HCC bevestigd.

We zagen dat ROS-vorming niet beperkt is tot enkel wel- of niet-genotoxische stoffen in HepG2-cellen, zoals ook waargenomen in een recente toxicogenomics studie in muizen [47]. We suggereren echter dat het verschil in werkingsmechanisme tussen oxidatieve stress inducerende genotoxische carcinogenen en niet-genotoxische carcinogenen de mate van oxidatieve stress inductie is ; bijv. exogene ROS vorming door metabolisme van genotoxische versus indirecte endogene vorming van ROS door cytochroom P450 activering, AhR binding, PKC activering en GSH uitputting [48, 49] veroorzaakt door niet-genotoxische carcinogenen.

Terwijl een groot aantal significante veranderingen in het transcriptoom, door oxidatieve stress geïnduceerd, sterk gecorreleerd werden aan toxiciteits- en kanker processen, leverde onze DNA methylatie en hydroxymethylering studies ons geen bewijs op dat deze epigenetische veranderingen, geïnduceerd door oxidatieve stress, bijdragen aan een carcinogeen profiel. Andere studies hebben echter aangetoond dat een verhoging van zowel 5mC en 5hmC modificaties gekoppeld zijn aan HCC en worden geacht een rol te spelen bij de initiële verandering van kanker-gerelateerde genexpressie in HCC ontwikkeling [36]. In tegenstelling lijkt in onze studies het grote aantal waargenomen veranderingen in DNA-methylatie eerder een adaptieve respons te zijn op de initiële oxidatieve stress-gerelateerde respons van het transcriptoom. Omdat methyltransferase

genen niet differentieel tot expressie komen tijdens blootstelling aan oxidanten, suggereren we dat deze adaptieve respons van het methyloom onafhankelijk gebeurt van genexpressie van verschillende DNMT enzymen. Een dergelijke DNMT-onafhankelijke adaptieve respons werd eerder waargenomen in rattenhersenen na acute stress door immobilisatie [50] alsook in een humane B-lymfoblast cellijn blootgesteld aan lage dosissen ioniserende straling [51]. Wijzigingen in het hydroxymethyloom, aan de andere kant, lijken niet op een dergelijke adaptieve respons ten opzichte van het transcriptoom, noch op carcinogeen-gerelateerde reacties. Deze veranderingen in 5hmC niveaus hadden slechts beperkte en eerder verwaarloosbare effecten op het transcriptoom.

Mogelijk kunnen in de humane *in vivo* situatie enkel toxische effecten op het niveau van het epigenoom ontstaan die leiden tot het ontstan van HCC en verdere progressie, door hogere en/of langdurige doses van ROS.

Als laatste vonden we dat functionele en transcriptoom reacties in HepG2 cellen verschillend waren dan die in Caco-2 cellen onder oxidatieve stress. Als we deze verkregen kennis toepassen op de fysiologische omstandigheden in de mens, suggereren we dat een bepaalde stof met oxidatieve stress-inducerende capaciteiten schade kan toebrengen aan meerdere doelorganen in het menselijk lichaam. De bepalende factor hierin is hoe deze stof wordt opgenomen en getransporteerd wordt doorheen het lichaam wat vervolgens kan leiden tot verschillende weefselspecifieke reacties.

Tot slot geven de resultaten besproken in dit proefschrift ons nieuwe moleculaire inzichten in tijdsafhankelijke oxidatieve stress-geïnduceerde reacties in het transcriptoom die correleren aan HCC ontwikkeling en hoe deze verschillen tussen celtypen. Veranderingen geïnduceerd in het epigenoom leverde echter geen bijdrage aan dit carcinogeen profiel, maar we toonden wel een belangrijke rol van het methyloom in het contra-reguleren van oxidatieve stress reacties van het transcriptoom eerder in de tijd om de normale cellulaire functie te herstellen. Door tijdreeks-analyses toe te passen konden we het HepG2 cel-specifieke oxidatieve stress genexpressieprofiel vinden dat bij kan dragen aan de verbetering van de risicoanalyse en kennis over werkingsmechanismen van toxicologische reacties die specifiek in de lever geïnduceerd worden door verschillende types oxidatieve stress-inducerende (niet-) levercarcinogenen. In tegenstelling tot wat is weergegeven in eerdere studies in ratten levers [24-26], laten we met het gebruik van een humane levercellijn in een vergelijkende studie met verschillende soorten (niet-) levercarcinogenen zien dat oxidatieve stress geen niet- genotoxisch carcinogeen-specifieke fenomeen is en kan daarom niet kan worden gebruikt om te discrimineren tussen genotoxische en niet-genotoxische carcinogenen. Tot slot, terwijl we een duidelijke correlatie en regulering tussen functionele eindpunten, het transcriptoom en het methyloom observeerden in reactie op oxidatieve stress, leken de veroorzaakte veranderingen in het hydroxymethyloom eerder op een gevolg van willekeurige processen.

Globaal gezien hebben deze resultaten bijgedragen tot het ontrafelen van onbekende oxidatieve stress gerelateerde mechanismen in de ontwikkeling van HCC. Daarnaast legt dit werk de basis voor een verdere verbetering van de risicobepaling van mogelijke levercarcinogenen. Dit kan dus nieuwe kansen creëren voor het bevorderen van preventieve maatregelen en vroegtijdige behandeling van HCC.

Toekomstige aanbevelingen

Nieuwe mechanistische informatie over oxidatieve stress reacties geïnduceerd door verschillende oxidatieve verbindingen is verkregen door het gebruik van HepG2-cellen. Ook een HepG2-specifieke oxidatieve stress genexpressieprofiel werd geïdentificeerd en toegepast voor een betere herkenning en mechanistisch begrip van oxidatieve stress-geïnduceerde cel schade door verschillende soorten levercarcinogenen. Het is echter belangrijk om te benadrukken dat slechts een beperkt aantal stoffen met slechts één enkele dosis in slechts één *in vitro* cel model bestudeerd zijn in dit proefschrift. Daarom is het belangrijk dat in de toekomst extra werk moet uitgevoerd worden in de validatie van deze resultaten met meer levercarcinogenen en stoffen die geen toxisch effect veroorzaken in de lever gevolgd door validatie in andere cel modellen.

Aangezien HepG2 cellen meerdere beperkingen hebben, zoals een lage expressie van belangrijke metabole en levertoxiciteit-gerelateerde genen en een ook over een afwijkend karyotype beschikken in vergelijking met normale levercellen, is validatie in andere *in vitro* levermodellen van belang. Primaire Humane Hepatocyten (PHH) worden beschouwd als het beste *in vitro* cel model voor leverstudies [52]. Het gebruik van deze cellen in toxiciteits-screenings is echter ook problematisch als gevolg van moeilijkheden bij de kweek procedures en interindividuele verschillen tussen leverdonoren. Bovendien maakt de beperkte beschikbaarheid van verse humane lever monsters het gebruik van PHH duur en belemmert daardoor het gebruik van deze cellen voor grote screeningsdoeleinden [53]. Onlangs zijn driedimensionale, organotypische celkweken, zoals lever sferoïden of organoiden [54], ontwikkeld. In sferoïden worden co-culturen van PHH of leverkanker cellijnen in combinatie met Kupffercellen gebruikt om een micro-weefsel te vormen, terwijl organoiden stamcellen zijn die afkomstig zijn van een humaan weefsel [54]. In beide modellen kunnen cellen zichzelf organiseren in de matrix op een manier die de *in vivo* situatie weerspiegelt [54, 55]. Over het algemeen bootsen deze 3D modellen de humane weefsels beter na dan de 2D cultuur cellen die zich in een monolaag bevinden omdat de cellulaire micro-omgeving die in de 3D-modellen gevormd wordt vaak een belangrijke rol speelt bij ziekteverloop en cellulaire reacties op geneesmiddelen [56, 57]. Samen met pluripotente stamcellen, die volledig kunnen worden omgezet in metabool actieve humane hepatocyten [58], zijn deze nieuwe *in vitro* modellen veelbelovend voor humane toxiciteit screening [59].

Naast de toepassing van deze nieuwe *in vitro* cel modellen, is het ook belangrijk om ook deze data van dergelijke nieuwe *in vitro* studies te verbinden met bestaande data uit klinische onderzoeken bij HCC patiënten, zoals wij hebben gedaan in onze studies met HepG2 cellen voor het voorspellen in welke mate oxidatieve stress-geïnduceerde mechanismen kunnen bijdragen aan de ontwikkeling van HCC [31].

Onze op het epigenoom-gebaseerde studies hebben ons voorzien van relevante en innovatieve informatie over adaptieve reacties van het methyloom op de oxidatieve stress reacties van het transcriptoom. Ten eerste, omdat DNA methylatie veranderingen slechts gedeeltelijk correleren met genexpressie veranderingen, zijn er ook andere cellulaire processen zoals microRNAs, eiwitfosforylatie, en acetylering of methylatie van histonen, die moeten worden onderzocht, omdat deze met DNA methylatie kunnen samenwerken om genexpressie te normaliseren in reactie op oxidatieve en alkylerende stress. Om een beter begrip van deze epigenetische processen en reacties op oxidatieve stress te verkrijgen, zullen bijkomende experimenten uitgevoerd en gecorreleerd moeten worden aan veranderde genexpressie. Bijvoorbeeld, inactiveren van de oxidatieve stress-gerelateerde transcriptiefactor NRF2 met behulp van interferentie RNA (siRNA of shRNA) kan ons ondersteunende informatie geven met betrekking tot onze hypothese over de verstoring van het samenspel tussen het transcriptoom en epigenoom - en zelfs het verlies van deze - in reactie op oxidatieve stress. Bovendien zijn onze microarray experimenten beperkt tot specifieke geannoteerde regio's, terwijl 'next-generation-sequencing' (NGS) aanvullende informatie biedt met betrekking tot basenparen specifieke data, zoals splicing varianten, niet-coderend RNA of nieuwe transcripten met een hogere specificiteit en sensitiviteit. Door het gebruik van deze geavanceerde 'omics techniek zijn we in staat om specifieke regio in het genoom te bepalen die mogelijk meer gevoelig zijn voor oxidatieve schade op verschillende tijdstippen en dit te vertalen naar relevante biologische informatie. Ook zal er meer inspanning nodig zijn om onze kennis over oxidatieve stress-geïnduceerde 5hmC aanpassingen te verhogen. Zo kan TET1 geïnactiveerd worden in een oxidatieve stress blootgestelde *in vitro* levercel model met behulp van siRNA of shRNA benaderingen om zijn rol in oxidatieve stress geïnduceerde 5hmC wijzigingen te evalueren [60].

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ADDENDUM

Valorisation

Relevance

The focus of this thesis was on gaining a better insight in the carcinogenic impact of oxidant exposure, with particular emphasis on hepatocellular carcinoma (HCC) using a human hepatoma cell line (HepG2). HCC is the predominant primary malignancy of the liver in most countries. It is the fifth most common cancer in men, and seventh among women, and overall the second leading cause of cancer related mortality in the world [1]. Together with the millions of estimated new cases each year, HCC inflicts a high social but also economic burden. Therefore, investing in prevention, early detection and treatment of this disease is of major importance.

Chronic liver disease due to hepatitis B virus (HBV) or hepatitis C virus (HCV) accounts for the majority of HCC cases in developing countries, while in Western Europe and Northern America other risk factors exist such as lifestyle in general (*e.g.*, obesity and diabetes) and exposure to compounds present in the environment, drugs or food [1, 2]. Prevention of HBV is possible by vaccination, while a HCV vaccine is until today not available but in development. The existence and development of such vaccines and the subsequent control of these viral infections, predicts a decrease in the rates of HCC, especially in developing countries [3]. However, as the contributions of HBV and HCV infections diminish, other risk factors present in the environment may become increasingly important as drivers of the future HCC incidence worldwide. Although these different inducers of HCC have different cellular targets and modes of action, their common feature is the formation of oxidative stress [4, 5]. Oxidative stress plays an important role in HCC initiation and progression and is even associated with intrinsic drug resistance [6]. Moreover, oxidative stress does not only induce mutations in DNA but can also induce changes in the DNA methylation status or inflict histone modifications which may further contribute to genomic instability and hepatocarcinogenesis [7-11].

Already decades ago, it is hypothesized that antioxidants can be used to scavenge these reactive oxygen species (ROS) and thus reduce damage. However, in large clinical trials [12-15], the effects of antioxidants could not be proven, on the contrary, they can even be harmful since ROS are also necessary to maintain normal cellular physiology [16]. For that reason, it is necessary to increase our understanding on the underlying molecular responses of the transcriptome and epigenome towards oxidative stress-induced damage and how this can be recognized, by for example specific gene signatures, to improve the risk assessment of new and existing chemicals, such as potential carcinogens and drugs.

Target groups

The knowledge obtained in this thesis can be used for further translational and applied research purposes, for instance for developing *in vitro* tests in the

pharmaceutical, food and chemical industry. These may increase the correctness of prediction, since an incorrect prediction of human safety increases the risk of admitting dangerous chemicals (in drugs, pesticides, food, *etc*…) to the market. On the other hand, incorrect prediction, due to differences in toxic responses between rodents and human, might also lead to the rejection of truly innocent compounds from the market, from which consumers can benefit, for instance as a drug or food additive.

In the pharmaceutical industry, before new drugs proceed into clinical trials, preclinical testing is performed, which mostly consists out of long-term animal experiments. If a drug does not induce adverse effects in these *in vivo* animal tests, the drug will be tested further in clinical trials using patients and healthy individuals. Roughly 20% of all newly developed drugs which precede this far in the pipeline of drug development are proven successful and become publically available [17]. This complete process of development and approval of drugs may cost up to US $800 million before they are released onto the market, what makes it a time-and money-consuming business [18]. Moreover, despite the existing toxicity testing procedures of new drugs, some drugs available on the market are identified to be hepatotoxic and can even induce HCC (*e.g.*, Bromfenac and Troglitazone). This can be induced by active metabolites or the formation of oxidative stress during metabolization of specific drugs, which makes hepatotoxicity one of the major reasons for drug withdrawal [2]. Also the lack of knowledge in epigenetic processes which can be induced by different oxidative stress-inducing compounds can contribute to such adverse outcomes in later phases of drug development. Additionally, many drugs are pharmacological hepatotoxic (=dose dependent, *e.g.*, Acetaminophen) which are unlimitedly available without a doctor's prescription [19]. This can have tremendous effects on population's health but also have a disastrous effect for a company's credibility and economic status. This indicates that toxicity testing in the pharmaceutical industry needs to be improved to reduce the number of falsely predicted compounds and subsequent economic and social costs.

The same applies to the food and chemical industry. Both have to follow strict rules and regulations in the development and use of their products to ensure human and environmental safety. Risks caused by chemicals (*e.g.*, pesticides, metals, …) and food additives (*e.g.*, food dyes, flavor enhancers, nanoparticles, …) have to be assessed, managed, and communicated to the customer by correctly labeling their products [20]. This process is strictly regulated by initiatives developed by the European Union, called REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) and OECD (Organization for Economic Co-operation and Development) [21, 22]. These authorities apply different programs to increase the safety of different chemicals. Because the current *in vivo* toxicity tests for human safety using rodents come along with a financial burden and ethical dilemmas, are not high-throughput and are questioned for their reliability towards the human situation [23], REACH and OECD also promote the development of alternatives for these animal tests. Mechanistic information discussed in this thesis can contribute to the development of new toxicity screening

methods and, since our results are obtained by *in vitro* cell models, it can contribute to the 3 R's (Reduction, Refinement and Replacement) in animal testing.

In general, the knowledge obtained in this thesis can contribute to the improvement of the risk assessment of such drugs, chemicals and food additives, but can also contribute to a more targeted approach in drug development in oxidative stress-driven cancers. By early prediction of toxicity induced via oxidative stress and better molecular understanding of this mechanism by compounds present in drugs, food and the environment, several future cases of HCC can be avoided which will lead to less human suffering and a lower economic burden.

Activities/products

Knowledge obtained in this thesis is mainly fundamental and describes mechanisms of oxidative stress in the development of HCC that can be used in more translational and applied research. Our results can create new opportunities for implementation of this knowledge in toxicological screening tests for investigating potential harmful or beneficial effects of new chemicals and drugs. In addition, compounds already present in the environment or pharmaceutical market can be re-evaluated for their oxidative stress-capacities over time which can lead to new information of possible hazardous effects for human or other organisms.

By using extensive time series-analysis for risk evaluation obtained from experiments on cell models in combination with transcriptomics and epigenomics, alternative approaches for animal studies in risk assessment can be developed. This can also service the further enrichment of new analytical tools and statistical approaches to make optimal use of the obtained data. Moreover, new treatment methods can be developed, since unravelling underlying pathways is the first step in the process of new drug development. However, since our knowledge is preliminary, more research is needed before applications of our results can be used to reduce the economic and social burden of HCC.

Innovation

This research focuses on oxidative stress mechanisms in the formation of liver toxicity and carcinogenesis. Different 'omics techniques such as transcriptomics and (hydroxy-)methylomics were used in combination with detection of phenotypic endpoints such as DNA damage and cell cycle distribution changes in order to unravel these specific oxidative stress-related mechanisms towards cellular damage. Since such oxidative stress-responses will differ in time, we used an innovative temporal analysis to examine time-dependent, sequential changes in gene expression and methylation to provide new insights in the responses towards oxidant challenge.

A combined temporal gene expression profile was identified for menadione and TBH exposure. This cluster consists out of 17 co-expressed genes that are involved in oxidative stress mechanisms and liver carcinogenesis (BIK, AKR1C2, GCLC, GCLM, GSR, LIF, RAP1GAP, SQSTM1, GCNT3, RRAS2, SLC7A11, ASF1A, ASKR1B10, FBXO30, AGPAT9, SRXN1, PTGR1). This specific oxidative stress related gene expression profile can be applied for a better recognition and mechanistic understanding of oxidative stress induced cell damage by chemicals. This gene set was used in Chapter 4 to identify genotoxic-, non-genotoxic- and non-carcinogens that have the ability to induce oxidative stress. We observed that a higher number of upregulated genes is associated with a higher genotoxic capacity of the compound. This demonstrates the relevance of this gene list as well as the use of time series-analysis to predict oxidative stress-inducing capacities of unknown compounds. This temporal gene expression profiles can be further developed by testing the expression of these particular genes in liver tissue and blood samples of HCC patients.

In addition, by combining transcriptomic and epigenomic data from the whole genome following oxidative stress, we were able to identify a counter-regulating property of the methylome towards the primary response of the transcriptome over time. These results further underline that toxicity screening has to be improved by emphasize on time series analysis but also on epigenetic responses towards different compounds.

These findings can eventually open up new doors for prevention and early treatment in HCC and possibly other chronic liver diseases.

Implementation

Apart from the economic aspect, societal value can be created from knowledge obtained in this thesis. Finding out more about the specific underlying oxidative stress mechanisms in hepatocarcinogenesis can ultimately lead to better prevention and early treatment methods. By using the previously discussed temporal gene expression profile as a diagnostic tool, potential patients can be screened for HCC in an early stage of disease. If the underlying mechanisms are really known, this will also lead to the reduction of side-effects of certain treatments, which is again beneficial for the patient.

As previously discussed, chemical safety is important and increasing the safety of compounds in drugs, environment and nutrition is a task of regulatory authorities such as the OECD. The OECD has recently launched a new program on the generation of Adverse Outcome Pathways (AOP) [24]. A better understanding of oxidative stress-related mechanisms of certain compounds can facilitate the construction of such AOPs. Possibly, an oxidative stress-specific AOP can be implemented in the future. The development of such *in vitro* tests can facilitate the further identification of key events in oxidative stress-related damage

induced by certain compounds. This will reduce the amount of laboratory animals, economic costs, but especially also the social impact of adverse drug effects that can be avoided by a better risk assessments.

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ADDENDUM

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ADDENDUM

Curriculum Vitae

List of Publications

Curriculum Vitae

Lize Deferme was born on September 8th 1986 in Heusden-Zolder, Belgium. After finishing her secondary school in the disciplines of “Sciences & mathematics” at the Sint-Fransiscus college in Heusden-Zolder, she started the bachelor study “Biomedical Sciences” in 2004 at Hasselt University in Diepenbeek, Belgium. In 2007, she continued her education with a Master’s program in Biomedical Sciences with a specialization in “Molecular Life Sciences” at Hasselt University. She did her graduation internship at the Flemish institute for technological research (VITO) in Geel, Belgium, under supervision of Dr. Hilda Witters. Here she investigated the swimming behavior of zebra fish embryo’s and larvae as an alternative method for neurotoxicity testing of chemicals. During her 2 master years, she also obtained certificates for radiation hygiene (expert level, 5b) and working with laboratory animals (Felaca C). In 2009, she graduated with magna cum laude at Hasselt University.

After her graduation in 2009, she started working as a research associate at the oncology research and development department of Johnson & Johnson in Beerse. Within a team of young researchers, she worked on the validation of potential targets in human prostate cancer using interference RNA.

In September 2010, she started as a PhD candidate at the department of Health Risk Analysis and Toxicology at Maastricht University, which is now the department of Toxicogenomics. Under supervision of Dr. Jacco Briedé and Prof.Dr. Jos Kleinjans, she worked on the results obtained in this thesis entitled: “Oxidative stress responses in Hepatocarcinogenesis: Unravelling the mechanisms using a Toxicogenomics approach”

List of Publications

Full Research papers:

Deferme L, Briede JJ, Claessen SMH, Jennen DG, Cavill R, Kleinjans JC (2013). Time series analysis of oxidative stress response patterns in HepG2: a toxicogenomics approach. Toxicology 306, 24-34

Deferme L, Briede JJ, Claessen SMH, Cavil R, Kleinjans JC (2015). Tissue-specific oxidative stress in cellular toxicity: a Toxicogenomics-based comparison between liver and colon cell models. Accepted Toxicology in Vitro

Deferme L, Wolters J, Jennen DG, Kleinjans JC, Briede JJ. Oxidative stress-induced mechanisms involved in genotoxic and non-genotoxic liver carcinogenesis. *Under review 2015*

Deferme L, Claessen SMH, Theunissen D, Wagner R, Kleinjans JC, Briede JJ. Methylation modifications at whole genome gene level induced by methyl and hydroxyl radicals. *Under review 2015*

Deferme L, Claessen SMH, Theunissen D, Wagner R, Kleinjans JC, Briede JJ. Oxidative stress induces dynamic changes in 5-hydroxymethylcytosine at random. *In preparation 2015*

Abstracts

Deferme L, Briede JJ, Claessen SM, Kleinjans JC, Temporal changes is radical formation and cellular responses after exposure to menadione and tert-butyl hydroperoxide in HepG2 cells. Poster presentation at workshop for Biomarkers of exposure and oxidative DNA damage from UKEMS/NVT/GUM meeting (2011) at Münster, Germany

Deferme L, Briede JJ, Claessen SM, Kleinjans JC, Temporal changes is radical formation and cellular responses after exposure to menadione and tert-butyl hydroperoxide in HepG2 cells. Poster presentation at the 16th Biennial meeting (2012) of Society for Free Radical Research International in London, UK

Deferme L, Briede JJ, Kleinjans JC. Oxidative stress in carcinogenesis: a Toxicogenomics based comparison between liver and colon carcinoma cells. Poster presentation at the PhD meeting of the Dutch Society of Toxicology (2011)

Deferme L, Briede JJ, Kleinjans JC. Oxidative stress in carcinogenesis: a Toxicogenomics based comparison between liver and colon carcinoma cells. Poster presentation at the 49th conference (2013) of the European society of Toxicology (Eurotox) in Interlaken, Switzerland

Deferme L, Wolters J, Briede JJ, Kleinjans JC Oxidative stress-induced mechanisms of toxicity and carcinogenesis by genotoxic and non-genotoxic compounds *in vitro*. Poster presentation at the 53th annual meeting (2014) of the Society of Toxicology (SOT) in Phoenix, Arizona, United States