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Identification of key pathways involved in the toxic response of the cyanobacterial toxin cylindrospermopsin in human hepatic HepaRG cells

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Abstract

The hepatotoxin cylindrospermopsin (CYN) has been involved in cases of poisoning in humans following ingestion. As its liver toxicity process is complex, we studied the transcriptomic profile of HepaRG cells exposed to CYN. The affected pathways were confirmed through the expression of key genes and the investigation of toxicity markers. In addition, CYP450 activities and cell redox homeostasis were investigated following acute and repeated exposure. CYN induced the down-regulation of genes involved in xenobiotic metabolism and cell cycle progression. There was cell cycle disturbance characterised by an accumulation of G1/S and G2/M cells and an increase in phospho-H3-positive cells. This was linked to the induction of DNA damage demonstrated by an increase in γH2AX-positive cells as well as an accumulation of sub-G1 cells indicating apoptosis but not involving caspase-3. While glutathione (GSH) content sharply decreased following acute exposure to CYN, it increased following repeated exposure, reflecting an adaptive response of cell redox homeostasis. However, our data also suggested that CYN induced the down-regulation of phase I and II metabolism gene products, and CYP450 activities were affected following both acute and repeated exposure to CYN. Our study indicated that repeated exposure of liver cells to low concentrations of CYN may affect their detoxification capacities.

Keywords: cylindrospermopsin, hepatotoxicity, transcriptomic, xenobiotic metabolism, cell cycle

Introduction

Cylindrospermopsin (CYN) is a 415-Da alkaloid toxin containing a tricyclic guanidine moiety linked to a hydroxyuracil ring. It is highly water-soluble due to its zwitterionic structure (Ohtani et al., 1992; Moreira et al., 2013). This toxin is produced by several terrestrial and freshwater cyanobacterial species such as *Raphidiopsis raciborskii* and *Hormoscilla pringsheimii* (Ohtani et al., 1992; Hawkins et al., 1997; Bohunická et al., 2015; Aguilera et al., 2018) and can be found all around the world from Antarctica to temperate regions (Kinnear, 2010; de la Cruz et al., 2013; Kleinteich et al., 2014). Unlike other cyanobacterial toxins, CYN can be actively released by intact cells into water during blooms, with the water fraction rising to up to 98% of the total amount produced (Chiswell et al., 1999; Shaw et al., 1999; Davis et al., 2014). Therefore, humans can be orally exposed to this toxin through drinking water. The ingestion of contaminated food or water due to CYN bioaccumulation in plants or aquatic organisms or during recreational water activities cannot be ruled out (Duy et al., 2000; Kinnear, 2010). Outbreaks of human poisoning implying CYN occurred following the consumption of contaminated water in Australia, and following the use of contaminated water for hemodialysis in Brazil (Bourke et al., 1983; Hawkins et al., 1985; Carmichael et al., 2001). The symptoms included visual disturbances, nausea, vomiting, gastro-enteritis and acute liver failure. Experiments performed on rodents revealed that CYN induced damage to the liver mainly through hepatocellular coagulative necrosis (Hawkins et al., 1985; Falconer et al., 1999; Griffiths and Saker, 2003). Considering the toxic effects of CYN and potential oral exposure in humans, this cyanobacterial toxin has been recognised as a potential public health risk (Chorus, 2005).

Since the liver has been shown to be the main target organ, CYN toxicity has been widely investigated *in vitro* using human hepatic cell lines (Froscio et al., 2009; Straser et al.,

2011; Kittler et al., 2016; Liebel et al., 2016) as well as primary rodent hepatocytes (Runnegar et al., 1994; Chong et al., 2002; Froscio et al., 2003; Lopez-Alonso et al., 2013). Several *in vitro* studies have reported that CYN induced a decrease in glutathione (GSH) levels and in antioxidant enzymes activities, leading to an increase in oxidative stress (Runnegar et al., 1994; Runnegar et al., 1995; Straser et al., 2013a; Liebel et al., 2015; Poniedzialek et al., 2015), and have also described the induction of genotoxic effects (Humpage et al., 2000; Bazin et al., 2010; Straser et al., 2011; Zegura et al., 2011). The mode of action of this toxin through the irreversible inhibition of eukaryotic protein synthesis has been demonstrated using acellular and cellular approaches (Terao et al., 1994; Froscio et al., 2001; Runnegar et al., 2002; Froscio et al., 2003). Xenobiotic metabolism has also been suggested as playing a role in CYN hepatotoxicity, especially through cytochromes P450 (Runnegar et al., 1995; Froscio et al., 2003; Bazin et al., 2010; Straser et al., 2013c; Liebel et al., 2015). However, the absence of CYN phase I-metabolite formation was recently demonstrated *in vitro* (Kittler et al., 2016). Therefore, some crucial pathways involved in hepatotoxic response to CYN still need to be identified.

In the present study we investigated the mechanisms of CYN-induced hepatotoxicity in the human hepatic cell line HepaRG. This model is similar to primary human hepatocytes regarding gene expression and the activities of the main phase I and II enzymes, transporters and nuclear factors as compared to other hepatic cell lines (Guillouzo and Guguen-Guillouzo, 2018). Considering potential repeated exposure to CYN, we performed experiments with acute as well as repeated exposure in HepaRG cells, since this model can remain functionally stable in a non-proliferative state for several weeks. After using a non-targeted approach with microarrays, we further confirmed the identified pathways by verifying the expression of key genes with RT-qPCR, investigating toxicity markers with a multiplex approach (high content analysis, HCA), and measuring cytochrome P450 (CYP450) activities.

Material and methods

Chemicals

William's E Medium, Minimum Essential Media without L-glutamine and phenol red (MEM), phosphate-buffered saline, penicillin and streptomycin were purchased from Gibco (Invitrogen, Cergy-Pontoise, France). Dimethylsulfoxide (DMSO), human insulin, L-glutamine, diclofenac, phenacetin, midazolam, bupropion, 4'-OH-diclofenac, acetaminophen, 1'-OH-midazolam, OH-bupropion and diclofenac-d4 (internal standard) were supplied by Sigma–Aldrich (Saint-Quentin-Fallavier, France). Hydrocortisone hemisuccinate was purchased from Upjohn Pharmacia (Guyancourt, France). Foetal calf serum (FCS) was supplied by Perbio (Brebières, France). CYN (kindly provided by Dr A. Humpage, Australian Water Quality Centre, Adelaide, Australia) was extracted and purified from an australian *Raphidiopsis raciborskii* culture (Norris et al., 2001), and prepared in sterile water. The "In Cytotox" kit was supplied by Biogenic (Perols, France). The "BCA Protein" kit, acetonitrile and methanol were purchased from Thermo Fisher Scientific (Courtaboeuf, France). Formic acid was supplied by Merck (Fontenay-sous-Bois, France). Deionised water was prepared using a Milli-Q system (Merck, Fontenay-sous-Bois, France).

Cell culture and differentiation

The human hepatic cell line HepaRG (Biopredic International, Rennes, France) was used at passages 14-20. Cells were grown in William's E Medium supplemented with 50

IU/mL penicillin, 50 µg/mL streptomycin, 2 mM L-glutamine, 5 µg/mL insulin, 25 µg/mL hydrocortisone hemisuccinate, and 10% FCS at 37°C in an atmosphere containing 5% CO_2. For subculture, cells were seeded in 75 cm^2 culture flasks and passaged every two weeks. For experimentation, cells were seeded at 2.5 x 10^4 cells/cm^2 (96-well plates for HCA and CYP450 activities, 48-well plates for cytotoxicity, 12-well plates for microarrays and reverse transcription quantitative PCR (RT-qPCR)). For cell cycle investigation, undifferentiated (proliferating) cells were used, while for the other assays, experiments were performed with differentiated cells. For differentiation, cells were incubated for two weeks before the addition of 2% DMSO to the culture medium for two more weeks. In all cases, the medium was changed three times a week.

Toxin exposure

Differentiated HepaRG cells were exposed to CYN in FCS-free medium for 24 h (acute treatment). CYN concentrations from 0.05 to 12.5 µM were tested for the neutral red uptake (NRU) assay. According to the results of the NRU assay, we chose CYN concentrations corresponding from low to 40% toxicity (0.8 µM for microarrays experiments, 0.5 to 2 µM for RT-qPCR assays, and 0.2 to 3.2 µM for CYP450 activities and GSH levels assays). Lower CYN concentrations (from 0.025 to 0.4 µM) were chosen for repeated exposure that was also performed on differentiated HepaRG cells for 14 days, with renewal of the medium (5% FCS and 1% DMSO) containing the toxin every two days. Undifferentiated HepaRG cells were exposed 24 h after seeding with 0.2 to 50 µM CYN in FCS-free medium for 24 h. This range of CYN concentrations was higher according to the results of Bazin et al. (2010). A vehicle control (sterile water) was included for each experiment. At least three independent

experiments and up to four and six independent experiments, for cytotoxicity and microarrays respectively, were performed.

Neutral red uptake assay

After 24 h of treatment with 0.05 to 12.5 µM CYN in differentiated HepaRG cells, the NRU assay was performed with the "In Cytotox" kit according to the manufacturer's instructions (Xenometrix, Allschwil, Switzerland). Absorbance was measured at 540 nm with a microplate-reading spectrofluorometer (FLUOstar OPTIMA, BMG Labtech, Champigny-sur-Marne, France). For each independent experiment, the median of the three technical replicates was expressed in relation to that of the vehicle control.

Microarray experiments and data analysis

Differentiated HepaRG cells were exposed for 24 h to a sub-toxic concentration of CYN (0.8 µM). Thereafter, total RNA was isolated, quantified and assessed for its integrity as previously described (Huguet et al., 2014). Probe preparation and hybridisation (using 4 X 44K Whole Human Genome 70-mer oligo-chips, G4112F, Agilent Technologies) with a completely randomised design were performed at the ANSES transcriptomic platform as previously described (Huguet et al., 2014) with the following modifications. Labelling was undertaken with either cyanine-3 CTP or cyanine-5 CTP, and absorbance was measured at 532 nm (for cyanine-3-labelled cRNA samples) or 635 nm (for cyanine-5-labelled cRNA samples).

Raw data extraction and quality control, Lowess normalisation and data filtering were performed as previously described (Huguet et al., 2014). This dataset, including the 29488

post-filtering probes, was labelled “filtered data” and has been deposited in National Center for Biotechnology information’s Gene Expression Omnibus through the accession number GSE123584. From the “filtered data”, the differentially expressed genes were selected at $P <$ 0.05 (Student t-test) and with a fold change (FC) greater than two (for “up-regulated genes”) or less than 0.5 (for “down-regulated genes”), leading to two distinct clusters. An analysis was performed for these clusters using the GoMiner application (http://www.discover.nci.nih.gov/gominer/index.jsp). Gene Ontology (GO) terms with a false discovery rate (FDR) score below 0.01 and an enrichment score above 1.5 were declared significant. An additional analysis was performed for the clusters with the Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatics resources (http://david.abcc.ncifcrf.gov). Using the DAVID Functional Annotation Tool, we visualised genes on Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway maps, selecting terms with a P-value below 0.005.

RT-qPCR

After 24 h of treatment with 0.5 to 2 µM CYN in differentiated HepaRG cells, total RNA was isolated, quantified and assessed for its integrity as described above, and a negative extraction control was included for the contamination assessment. Reverse transcription, qPCR and data analysis were performed as previously described (Huguet et al., 2014), with the following modifications and applying the guidelines for qPCR assay design and reporting (Bustin et al., 2009). For primers, their design and the *in silico* analyses of their specificity were performed together, using the Basic Local Alignment Search Tool for primers (http://www.ncbi.nlm.nih.gov/BLAST/) with, for each gene, at least one primer designed to span an exon-exon junction. All primers were purchased from Sigma-Aldrich (Lyon, France).

The *actb* gene was chosen as a reference gene since it did not exhibit any significant variation in expression among all the samples. Additional information regarding the target genes and oligonucleotide primers is listed in Table S1. The qPCR reactions were performed with 0.5 ng cDNA, and the thermal cycling conditions were 94°C for 10 min, followed by 40 cycles of denaturation at 94°C for 15 s, annealing at the determined temperature for 15 s, and polymerisation at 72°C for 30 s. For the contamination assessment, the results revealed that the ΔCqs of the samples were at least 5.5 as compared to the various controls (extraction, RT, no-reverse transcriptase, and qPCR). Values were presented as arbitrary units.

Measurement of CYP450 activities

Following acute (0.2 to 3.2 µM CYN) and repeated (0.025 to 0.4 µM CYN) exposure in differentiated HepaRG cells, CYP450 activities were measured as previously described (Ferron et al., 2016). Briefly, cells were incubated at 37°C for 120 min with a cocktail of specific CYP450 substrates (93 µM diclofenac, 100 µM phenacetin, 5µM midazolam, and 94.6 µM bupropion) in MEM, and metabolites were quantified by LC/MS-MS. CYP450 activities were calculated as pmol of metabolite formation per min and per mg of protein.

High content analysis of multiparametric toxicity

GSH levels in differentiated HepaRG cells

Following acute (0.2 to 3.2 µM CYN) and repeated (0.025 to 0.4 µM CYN) treatments, differentiated HepaRG cells were incubated at 37°C for 30 min with 80 µM monochlorobimane (Thermo Fisher Scientific, M-1381MP) and 1/500 CellMask™ Deep Red stain (Thermo Fisher Scientific, H32721) in MEM. Following washes in MEM, plates were

scanned with a Thermo Scientific Arrayscan VTI HCS Reader associated with a live cell chamber, and 10 fields were analysed per well. Cell counting was performed using CellMask™ Deep Red staining. Using the Spot Detector module of the BioApplication software, the GSH conjugate of monochlorobimane was quantified in the cytoplasm. Spot average intensity was determined and expressed in relation to that of the vehicle control.

Cell cycle progression, genotoxicity and apoptosis in undifferentiated HepaRG cells

Since differentiated HepaRG cells are quiescent, additional experiments on the modification of cell cycle progression by CYN were performed with undifferentiated HepaRG cells. After 24 h of treatment with 0.2 to 50 µM CYN, immunofluorescence was performed as previously described (Ferron et al., 2016), with the following modifications. Antibodies were purchased from Abcam (Cambridge, UK) and prepared at 1/1000 (except for Anti-Active Caspase-3, prepared at 1/200): Anti-γH2AX Ser139 Mouse Monoclonal Antibody (ab26350), Anti-active Caspase-3 Rabbit Monoclonal Antibody (ab2302), Anti-Phospho-H3 S10 Rabbit Antibody (ab5176), Anti-Rabbit IgG (H+L) DyLight 488 Goat Antibody (ab96891), and Anti-Mouse IgG (H+L) DyLight 550 Goat Antibody (ab96876). Using nuclear DAPI staining and the Cell Cycle Analysis module of the BioApplication software, cells were classified in the different cell cycle phases and expressed in relation to the total cell number. Active caspase-3 (for apoptosis) was quantified in the whole cell, while γH2AX (for genotoxicity) and phospho-H3 (for cell cycle progression) were quantified in the nuclei. The images were analysed using the Target Activation module of the BioApplication software. Cells were defined as positive when their average intensity exceeded a threshold of

two standard deviations when compared to the average intensity of the vehicle control cells, and positive cells were expressed in relation to the total cell number.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism software (version 5.0, GraphPad Software Inc., La Jolla, CA). For cytotoxicity, cell counts, and GSH measurements, data were analysed using the One-sample t-test with "100" as the theoretical mean. Means were declared significantly different from 100 at $P < 0.05$, and trends ($P < 0.1$) were included. For RT-qPCR results, CYP450 activities, and the rest of the HCA data, an analysis of variance was performed. When the concentration effect was significant ($P < 0.05$), the values were compared using Dunnett's test. Differences were declared significant at $P < 0.05$. The values presented are means ± SEM.

Results

Cytotoxicity and transcriptomic profile

A concentration-dependent decrease in viability was observed in differentiated HepaRG cells after 24 h of exposure to CYN, and an IC_{50} of 4.1 ± 0.5 µM was determined (Figure 1). No decrease in cell viability was observed up to 0.8 µM CYN, but cytotoxicity reached 100% with 12.5 µM CYN ($P < 0.05$).

Based on these results, a sub-toxic concentration of 0.8 µM CYN was selected for microarray experiments in differentiated HepaRG cells. From the "filtered data", we

determined 1061 up-regulated genes and 1055 down-regulated genes (respectively FC > 2 or < 0.5, and *P*-value < 0.05) following CYN treatment. The biological and molecular processes affected by CYN were addressed using the GoMiner application and the DAVID Functional Annotation Tool.

For the 1061 up-regulated genes, 18 biological processes, 12 cellular components and four molecular functions were significantly enriched (Table S2). Of the biological processes, 12 were related to the modification of non-coding RNA such as tRNA and rRNA, while the others were associated with ribonucleoprotein complex biogenesis and translation. The cellular components were mainly related to the nucleus, cajal body and ribosome, and the molecular functions were related to RNA binding and RNA modifications. Therefore, our data suggested that the main proteins encoded by the up-regulated genes belong to ribonucleoprotein complexes and are involved in aspects of RNA maturation such as splicing and translation occurring respectively inside the nucleus and along the ribosomal machinery. An additional analysis using the DAVID Functional Annotation Tool highlighted three significant terms (data not shown) with two already having been identified using GoMiner. The first term, "RNA degradation", referred to the exosome involved in RNA maturation occurring in the nucleus. This complex is formed from core proteins (such as EXOSC2, EXOSC3, EXOSC7 and EXOSC8) and is associated with several factors such as DCP1B, LSM7, MPHOSPH6 and DCPS. The second term, "spliceosome", described a dynamic complex of small nuclear ribonucleoproteins involved in pre-mRNA splicing events and containing various factors such as PRPF38A and SNW1. These data suggested that CYN modified the gene expression of proteins involved in RNA modification and maturation prior to translation.

The 1055 down-regulated genes gave a list of 64 biological processes, 38 cellular components and 25 molecular functions (Table S3). Of the biological processes, 23 were

related to mitosis while 18 were involved in cellular metabolism for lipids, alcohols and organic acids (Figure 2). The majority of the cellular components were related firstly to microtubules and chromosomes and secondly to vesicles, lysosomes and endoplasmic reticulum. The 25 molecular functions were mainly related to the activities of enzymes such as oxidoreductase and transferase (Table S3). Proteins encoded by genes from the down-regulated cluster are involved in both cell division and the activity of metabolic enzymes. Using the DAVID Functional Annotation Tool, the analysis described 12 significant terms (Table S4) also described with GoMiner, mostly corresponding to xenobiotic-metabolising enzymes such as CYP450, UDP-glycosyltransferase, glutathione S-transferase (GST) and alcohol dehydrogenase. Another significant term, "cell cycle", including coding genes for CCNA2, CDK1 and PCNA, was also outlined. Our results suggested that the xenobiotic metabolism and cell cycle progression of HepaRG cells are affected after exposure to CYN.

Validation of CYN-induced gene expression

The expression levels of 18 key up- and down-regulated genes involved in the processes detailed above were further evaluated by RT-qPCR in differentiated HepaRG cells exposed to CYN concentrations ranging from low to 25% toxic (0.5 to 2 µM). The results are summarised in Table 1. For all genes, a concentration-dependent response for gene expression was obtained. For RNA degradation, the expression levels of *exosc2* and *mphosph6* increased significantly to respectively 3.9- and 2.3-fold greater than that of the control ($P < 0.01$). A similar pattern for *prpf38a* and *snw1* (spliceosome) was observed: respectively 2.4-fold greater than that of the control ($P < 0.05$) and two-fold greater (but not significant). Regarding the target genes of the detoxification process (*adh1b*, *cyp2c8*, *gstm2*, *scd*, *ugt2b4*, *ugt2b15* and *ugt2b17)*, their expression levels were strongly affected, with a sharp decrease ranging from

five- to 16.4-fold lower than that of the control ($P < 0.01$) at the highest CYN concentration. Lastly, for cell cycle progression, expression levels for *ccna2*, *cdk1*, *cdkn2c*, *pcna*, *pkmyt1* and *ttk* also significantly decreased ($P < 0.05$ except for *cdk1*) for the highest concentration (from 3.5- to 12.1-fold lower than that of the control).

Modification of GSH levels and CYP450 activities

GSH levels and CYP450 activities were evaluated in differentiated HepaRG cells after acute as well as repeated exposure to CYN in order to assess the adaptive response of the cells. Acute CYN treatment induced a concentration-dependent decrease in GSH levels compared to the control, while no decrease in cell counts was observed (Table 2). Under the repeated-exposure scenario, we observed a sharp increase in GSH levels (up to three-fold at 0.2 µM), which were then halved when there was 30% toxicity.

Regardless of the exposure scenario (acute or repeated), a sharp decrease in CYP450 activities was observed with low CYN concentrations (Table 3). Acute CYN exposure reduced CYP2B6, CYP2C9 and CYP3A4 activities by more than half compared to the control. Following 14 days of repeated exposure to 0.1 µM CYN, CYP2C9 and CYP3A4 activities were also reduced (by -59% and -91%, respectively).

Cell cycle progression, genotoxicity and apoptosis quantified by high content analysis in undifferentiated HepaRG cells

A concentration-dependent decrease in cell counts was observed after 24 h of CYN treatment, and an IC_{50} of 5.9 ± 0.2 µM was determined (Table 4). The toxicity profile observed by cell counting in undifferentiated HepaRG cells was close to that obtained by

NRU in differentiated HepaRG cells (IC_{50} of 4.1 ± 0.5 µM). Whenever toxicity was observed (3.2 µM), an increase in S and G2/M cells and a decrease in G0/G1 cells were detected concomitantly (Figure 3). The accumulation of cells in the G2/M phase was confirmed by the increase in phospho-H3-positive cells above 6.2 µM CYN (Table 4). The proportion of sub-G1 cells also increased from 12.5 µM CYN, suggesting an apoptotic effect. Further investigations with γH2AX and caspase-3 showed that, although a concentration-dependent increase in γH2AX-positive cells was observed with CYN treatment, no increase in caspase-3-positive cells was detected.

Discussion

Exposing HepaRG cells to CYN induced the down-regulation of genes involved in xenobiotic metabolism and cell cycle progression. There was cell cycle disturbance linked to the induction of DNA damage, and CYP450 activities were affected following both acute and repeated exposure to CYN.

For the first time to our knowledge, experiments were performed using a non-targeted approach in order to evaluate the modulation of gene expression by CYN. The analysis of the transcriptomic profile of HepaRG cells treated with a low concentration of CYN revealed that genes associated with RNA maturation were up-regulated. Some of these genes code for enzymes forming the exosome, a complex of proteins participating in RNA quality-control mechanisms before translation (Chlebowski et al., 2013). The increased expression levels of EXOSC2, a component of the exosome core, and MPHOSPH6, a co-factor of the exosome, were confirmed by qPCR. The up-regulation of some genes coding for spliceosome proteins involved in pre-mRNA splicing (Hoskins and Moore, 2012; Chen and Moore, 2014) was

confirmed by qPCR experiments for two of these factors, PRPF38A and SNW1. Using another cell model, differentiated Caco-2 cells, we previously reported an increase in the expression level of the DDX20 gene, a member of the DEAD-box family involved in RNA maturation, following CYN treatment (Huguet et al., 2014). Therefore, RNA maturation before translation is likely a common process affected by CYN for various cell models.

Several pathways, including phase I and II metabolism as well as cell cycle progression, were affected following acute exposure to CYN as also previously reported in HepG2 cells (Straser et al., 2013c). Regarding cell cycle progression, we observed a cell cycle arrest demonstrated by an accumulation of cells in the G2/M phase and an increase in phospho-H3-positive cells, markers of aneuploidy and mitotic block (Bryce et al., 2016). There was also an accumulation of cells in the S phase, combined with a decrease in the expression of *pcna* and *cdkn2c*, a gene controlling the G1/S cell transition. Cell cycle arrest had been previously reported in HepG2 cells exposed to CYN, and was characterised by a decreased amount of Ki67, increased expression of *cdkn1a*, decreased expression of *pcna* and *cdkn2c*, and an accumulation of cells in the S phase (Bain et al., 2007; Straser et al., 2013b; Straser et al., 2013c). This alteration of cell cycle progression originated in DNA damage induced by CYN as reported in various cell models (Bain et al., 2007; Lankoff et al., 2007; Straser et al., 2013b; Straser et al., 2013c; Poniedzialek et al., 2014). Indeed, several authors have reported the induction of DNA damage by CYN in HepaRG and HepG2 cells using different methods (micronucleus, comet and γH2AX assays) (Bazin et al., 2010; Straser et al., 2011; Zegura et al., 2011; Straser et al., 2013a; Straser et al., 2013b). In our study, the increased proportion of γH2AX-positive cells also indicated DNA damage. However, while CYN was shown to also affect the expression of genes involved in apoptosis and DNA damage repair in HepG2 cells (Bain et al., 2007; Straser et al., 2013c), we did not observe such effects in HepaRG cells. The increase in the sub-G1 population suggested an apoptotic effect of CYN. While caspase-

dependent apoptosis was reported in primary rodent hepatocytes exposed to CYN (Lopez-Alonso et al., 2013), this effect was not observed with HepaRG in our study, nor in HepG2 (Straser et al., 2013a). CYN-induced HepaRG cell apoptosis needs to be confirmed, and pathways not involving caspase-3 should be investigated.

We further examined whether the observed effects may have been related to the disturbance of cell redox homeostasis. After acute exposure of hepatic cells to CYN, several studies have reported the inhibition of GSH synthesis (decrease in γ-glutamylcystein synthase (γGCS) activity and in GSH levels) leading to oxidative stress due to a decrease in reactive oxygen species scavenging (Runnegar et al., 1994; Runnegar et al., 1995; Runnegar et al., 2002; Gutierrez-Praena et al., 2011b; Lopez-Alonso et al., 2013; Straser et al., 2013a; Liebel et al., 2015). We confirmed a strong decrease in GSH levels after acute exposure to low CYN concentrations in differentiated HepaRG cells, but without any modulation of gene expression involved in GSH metabolism. On the contrary, it was shown that γGCS activity and GSH levels increased following CYN exposure in intestinal Caco-2 cells and endothelial HUVEC cells (Gutierrez-Praena et al., 2012a, 2012b), suggesting a difference in cell sensitivity to CYN depending on the model used (Pichardo et al., 2017). Hepatic-derived cell lines were the most sensitive to CYN as compared to other models (Froscio et al., 2009). Compared to acute CYN exposure, GSH levels in differentiated HepaRG cells increased following repeated exposure to low CYN concentrations. Similarly, *in vivo* experiments in tilapia reported an increase in liver γGCS activity only after prolonged exposure to CYN (seven and 14 days) (Gutierrez-Praena et al., 2011a; Guzman-Guillen et al., 2014). In this case, an adaptive response through increased GSH production may prevent the oxidative stress caused by the toxin.

Our transcriptomic analysis also revealed the down-regulation of phase I and II metabolic enzymes, which was confirmed by qPCR for some of these. Literature data have

reported strong variations in the gene expression of xenobiotic-metabolising enzymes with up- or down-regulation (Zegura et al., 2011; Straser et al., 2013c; Rios et al., 2014). These discrepancies may have been due to the different methods and chemistries used for the detection of changes in gene expression, as suggested by Straser et al. (Straser et al., 2013c). The down-regulation of metabolic enzymes reported in our study was supported by the decrease in CYP450 activities observed after acute as well as repeated exposure of HepaRG cells to CYN. Similar results were obtained with a decreased amount of total hepatic microsomal CYP450 observed in mice (Terao et al., 1994) in addition to decreased GST activity in the liver of tilapia after 14 days of exposure (Rios et al., 2014). Repeated exposure to very low concentrations of CYN in hepatic cells could affect their detoxification capacities and probably other hepatic functions such as lipid and hormonal metabolism.

Conclusion

Acute exposure of differentiated HepaRG cells to a low concentration of CYN resulted in the up-regulation of gene products involved in RNA maturation as well as the down-regulation of those involved in xenobiotic metabolism and cell cycle progression. We confirmed many effects of CYN already observed by previous studies, but we highlighted a probably non-caspase-3-dependent apoptotic effect, and therefore this pathway needs to be studied. GSH metabolism was affected after acute exposure, but cell adaptation occurred when exposure was repeated. On the other hand, CYP450 activities were reduced with both acute and repeated exposure to CYN, combined with lower gene expression after acute exposure. Future research should focus on health hazard in case of prolonged exposure

through cell redox homeostasis and detoxification capacities (notably antioxidant enzymes and phase II metabolism).

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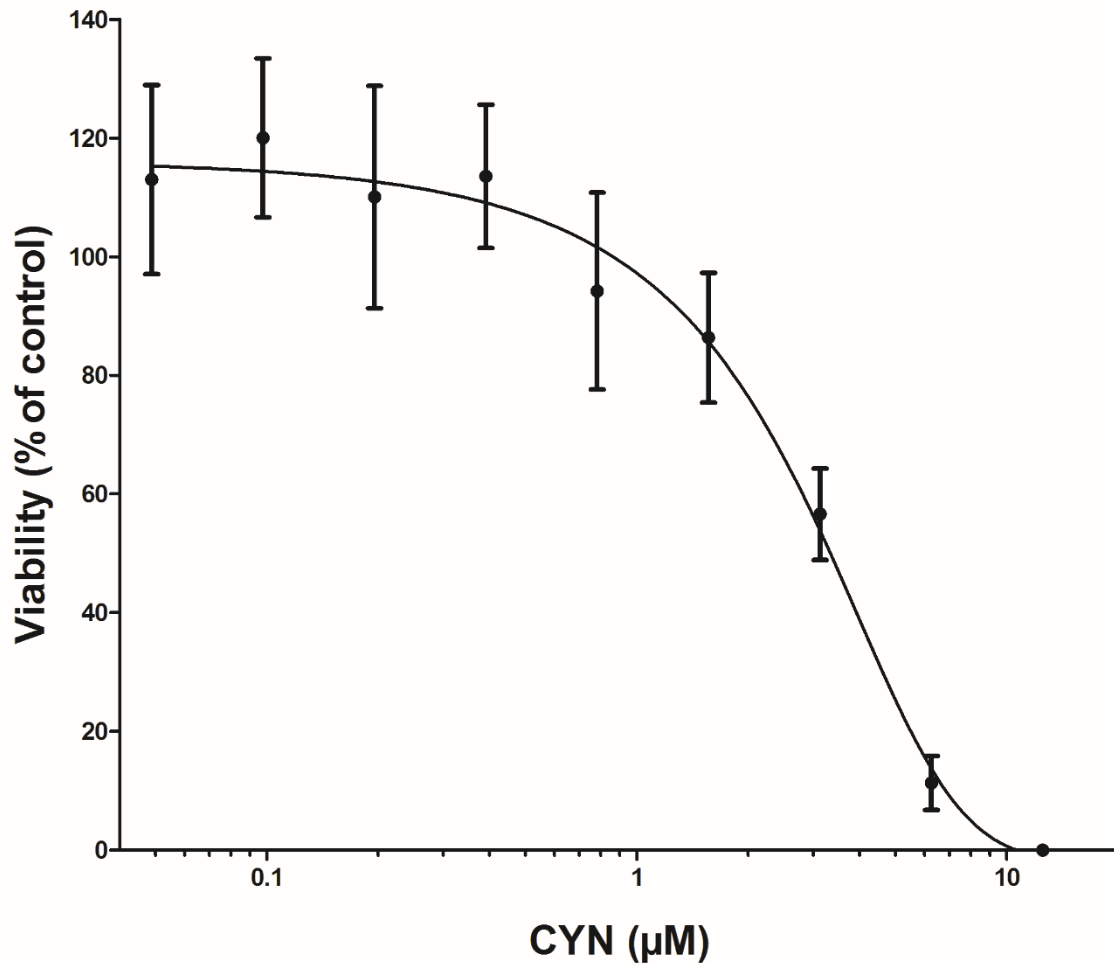

Fig. 1. Cytotoxicity in differentiated HepaRG cells after 24 h exposure to CYN. Cytotoxicity was measured by the NRU assay. Values are presented as means ± SEM and expressed as percentages of the vehicle control. Four independent experiments were performed.

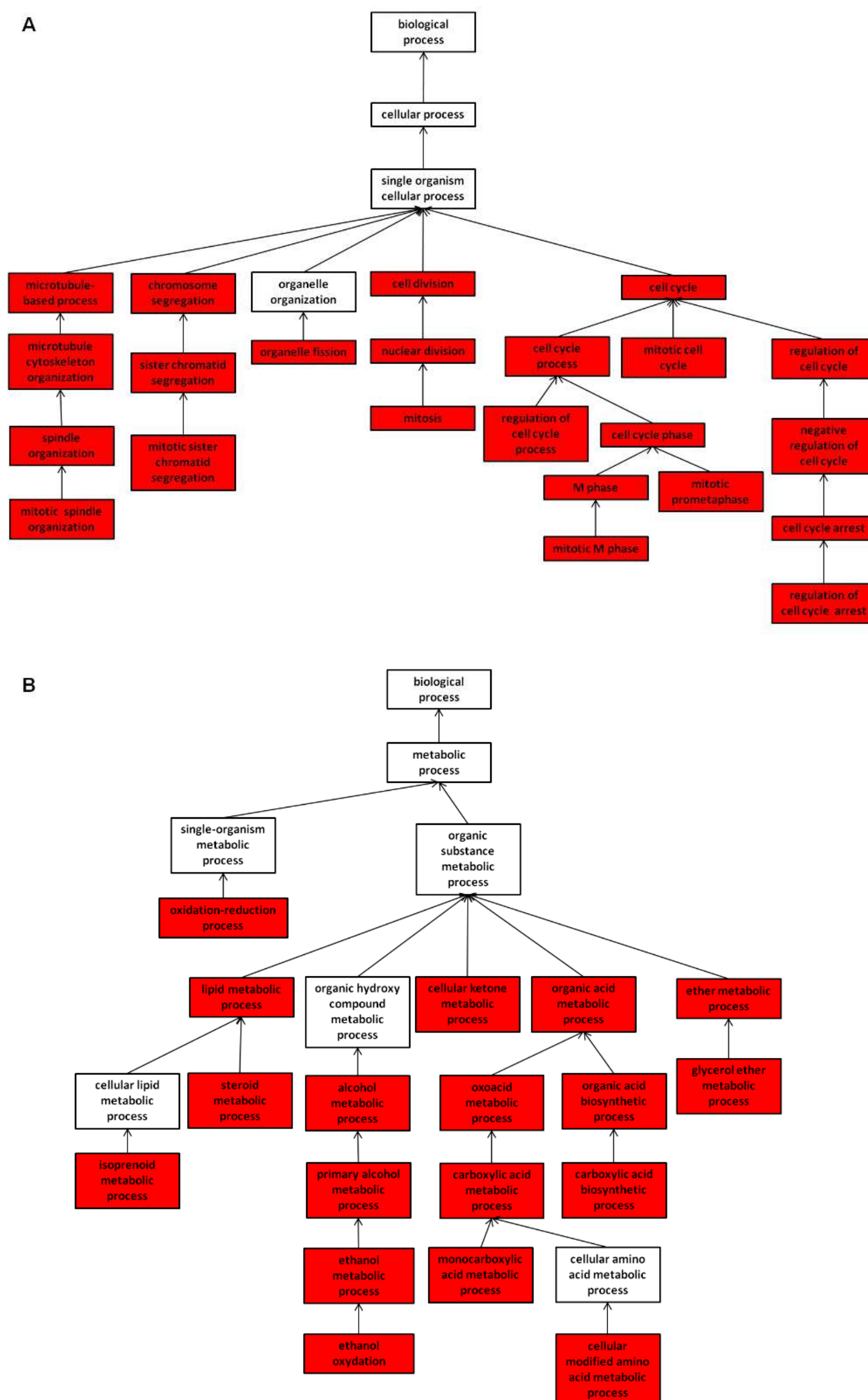

Fig. 2. Tree of the biological processes down-regulated in differentiated HepaRG cells after 24 h exposure to CYN. The 1055 down-regulated genes after 24 h of exposure to 0.8 µM CYN were annotated within 64 biological processes (GO terms) with an enrichment score above 1.5. The tree shows some of the 64 enriched GO terms with 23 biological processes related to the cell cycle (A), and 18 biological processes related to metabolism (B). The GO terms had a false discovery rate score below 0.01 (in red); the white GO terms were ancestors.

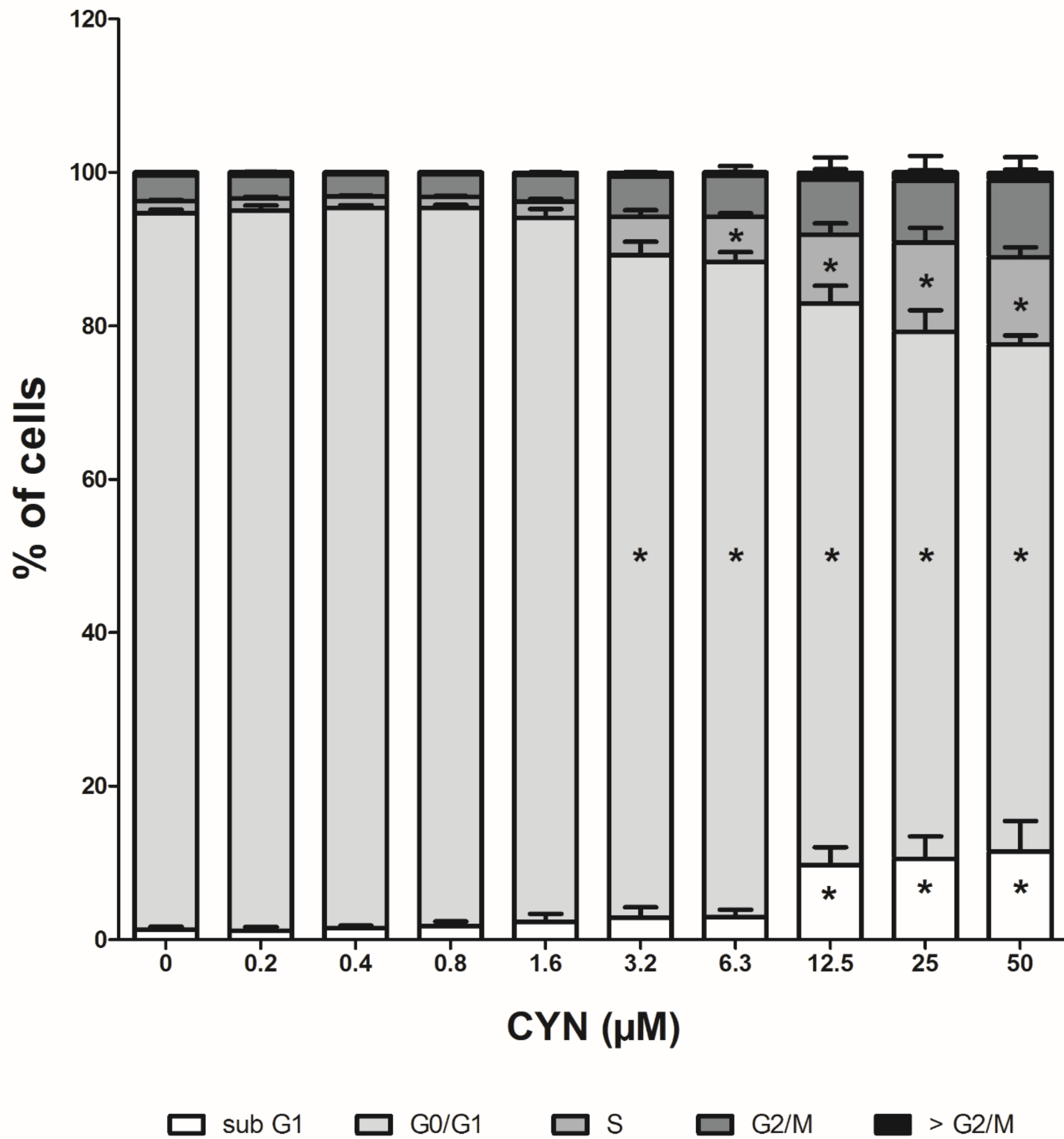

Fig. 3. Cell cycle progression in undifferentiated HepaRG cells after 24 h exposure to CYN. The classification of cells in the different cell cycle phases was determined using nuclear DAPI staining and is expressed in relation to the total cell number (>G2/M correspond to polyploid cells). Values are presented as means ± SEM. Three independent experiments were performed. *: values significantly different from the vehicle control ($P < 0.05$).

Table 1. Relative gene expression in differentiated HepaRG cells after 24 h of exposure to CYN. The genes have been taken from the transcriptome analysis. Values are presented as means ± SEM and have been normalised to the *actb* reference gene. Three independent experiments were performed.

Gene	Control	0.5 µM	1 µM	2 µM
		RNA degradation		
exosc2	0.23 ± 0.03	0.50 ± 0.07	0.71 ± 0.16[a]	0.89 ± 0.05[b]
mphosph6	0.58 ± 0.05	1.12 ± 0.07[a]	1.16 ± 0.16[a]	1.32 ± 0.12[b]
		Spliceosome		
prpf38a	0.76 ± 0.10	1.24 ± 0.17	1.26 ± 0.07	1.79 ± 0.31[a]
snw1	0.44 ± 0.04	0.67 ± 0.06	0.75 ± 0.16	0.90 ± 0.07
		Detoxification process		
adh1a	1.61 ± 0.36	0.81 ± 0.12	0.62 ± 0.11[a]	0.45 ± 0.17[a]
adh1b	3.17 ± 0.83	1.02 ± 0.13[a]	0.34 ± 0.05[b]	0.23 ± 0.06[b]
cyp2c8	1.97 ± 0.60	1.17 ± 0.14	0.41 ± 0.04[a]	0.12 ± 0.03[b]
gstm2	1.52 ± 0.20	1.33 ± 0.12	0.64 ± 0.11[b]	0.30 ± 0.08[c]
scd	1.57 ± 0.14	1.09 ± 0.26	0.73 ± 0.17[a]	0.28 ± 0.11[b]
ugt2b4	3.20 ± 0.48	1.81 ± 0.17[a]	0.80 ± 0.14[c]	0.21 ± 0.04[c]
ugt2b15	2.97 ± 0.46	1.87 ± 0.08[a]	1.03 ± 0.10[b]	0.47 ± 0.07[c]
ugt2b17	2.72 ± 0.48	1.68 ± 0.10[a]	0.93 ± 0.06[b]	0.54 ± 0.02[c]
		Cell cycle		
ccna2	1.47 ± 0.33	0.71 ± 0.17	0.33 ± 0.22[a]	0.14 ± 0.06[b]
cdk1	2.04 ± 0.65	1.11 ± 0.30	0.66 ± 0.30	0.30 ± 0.12
cdkn2c	3.02 ± 0.54	1.33 ± 0.13[b]	0.48 ± 0.11[c]	0.25 ± 0.04[c]
pcna	1.66 ± 0.31	1.30 ± 0.40	0.74 ± 0.15	0.47 ± 0.06[a]
pkmyt1	3.13 ± 0.42	1.40 ± 0.26[b]	0.87 ± 0.30[b]	0.51 ± 0.15[c]
ttk	2.74 ± 0.73	1.41 ± 0.43	0.63 ± 0.30[a]	0.47 ± 0.23[a]

[a] $P < 0.05$, [b] $P < 0.01$ and [c] $P < 0.001$: significantly different from the control group.

Table 2. GSH levels in differentiated HepaRG cells after acute (24 h) and repeated (14 days) exposure to CYN. Values are presented as means ± SEM and expressed as percentages of the vehicle control set to 100. Three independent experiments were performed.

Acute exposure				0.2 µM	0.4 µM	0.8 µM	1.6 µM	3.2 µM
Cell count				103 ± 2	100 ± 1	103 ± 1	103 ± 9	89 ± 13
GSH content				111 ± 12	106 ± 18	79 ± 22	31 ± 8[a]	4 ± 1[b]
Repeated exposure	**0.025 µM**	**0.05 µM**	**0.1 µM**	**0.2 µM**	**0.4 µM**			
Cell count	93 ± 6	92 ± 4	93 ± 2[a]	90 ± 1[a]	70 ± 5[a]			
GSH content	136 ± 27	167 ± 17	188 ± 33	302 ± 66	53 ± 21			

[a] $P < 0.05$ and [b] $P < 0.01$: significantly different from 100.

Table 3. Cytochrome P450 activities in differentiated HepaRG cells after acute (24 h) and repeated (14 days) exposure to CYN. The activities of cytochrome P450 enzymes were measured by detecting OH-bupropion for CYP2B6, 4'-OH-diclofenac for CYP2C9, and 1'-OH-midazolam for CYP3A4. Values are expressed as pmol/min/mg protein and presented as means ± SEM. Three independent experiments were performed.

Acute exposure	Control				0.2 µM	0.4 µM	0.8 µM	1.6 µM	3.2 µM
CYP2B6	6 ± 0.3				8 ± 0.4	8 ± 0.3	5 ± 1.1	2 ± 0.2[b]	< LOQ
CYP2C9	26 ± 3				29 ± 3	29 ± 6	23 ± 2	15 ± 2	11 ± 3[a]
CYP3A4	58 ± 5				62 ± 7	57 ± 3	50 ± 6	28 ± 5[b]	18 ± 4[c]
Repeated exposure	Control	0.025 µM	0.05 µM	0.1 µM	0.2 µM	0.4 µM			
CYP2C9	163 ± 42	242 ± 96	98 ± 10	66 ± 16	< LOQ	< LOQ			
CYP3A4	223 ± 58	161 ± 71	40 ± 10	21 ± 3[a]	< LOQ	< LOQ			

[a] $P < 0.05$, [b] $P < 0.01$ and [c] $P < 0.001$: significantly different from the control group.

Table 4. Cell cycle arrest, genotoxicity and apoptosis in undifferentiated HepaRG cells treated for 24 h. Cell count values are expressed as percentages of the vehicle control. Phospho-H3, γH2AX and caspase-3 cells were defined as positive (see Material and methods section). Values are presented as means ± SEM. Three independent experiments were performed.

	Control	0.2 µM	0.4 µM	0.8 µM	1.6 µM	3.2 µM	6.2 µM	12.5 µM	25 µM	50 µM
Cell count	100 ± 0	98 ± 1	100 ± 3	97 ± 1	89 ± 8	80 ± 9	53 ± 2[b]	18 ± 2[c]	14 ± 1[c]	10 ± 1[c]
Phospho-H3	1.7 ± 0.2	1.9 ± 0.3	2.2 ± 0.4	2.0 ± 0.4	4.0 ± 1.0	7.7 ± 2.1	30.5 ± 5.7[c]	30.1 ± 8.7[c]	24.6 ± 6.9[c]	16.9 ± 4.3
γH2AX	2.0 ± 0.1	1.6 ± 0.1	1.5 ± 0.1	1.4 ± 0.1	1.9 ± 0.2	8.2 ± 3.6	16.7 ± 3.3	31.9 ± 8.1[b]	28.8 ± 8.5[b]	25.5 ± 7.5[a]
Caspase-3	3.6 ± 0.2	4.9 ± 0.5	4.3 ± 0.9	3.3 ± 0.6	1.5 ± 0.3	1.4 ± 0.2	4.1 ± 1.0	6.7 ± 1.6	6.4 ± 1.7	6.7 ± 1.6

[a] $P < 0.05$, [b] $P < 0.01$ and [c] $P < 0.001$: significantly different from the control group.