UNIVERZA V LJUBLJANI
BIOTEHNIŠKA FAKULTETA

Alja ŠTRASER

GENOTOKSIČNO DELOVANJE CIANOBAKTERIJSKIH TOKSINOV NA HUMANE CELICE V POGOJIH *in vitro*

DOKTORSKA DISERTACIJA

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GENOTOXIC ACTIVITY OF CYANOBACTERIAL TOXINS IN HUMAN CELLS *in vitro*

DOCTORAL DISSERTATION

Ljubljana, 2013

Discovery consists in seeing what everyone else has seen
and thinking what no one else has thought.
Albert Szent-Gyorgi

Doktorska disertacija je zaključek podiplomskega študija bioloških in biotehnoloških znanosti s področja genetike na Biotehniški fakulteti Univerze v Ljubljani. Raziskovalno delo je bilo opravljeno na Nacionalnem inštitutu za biologijo, v laboratorijih Oddelka za genetsko toksikologijo in biologijo raka.

Na podlagi statuta Univerze v Ljubljani ter po sklepu senata Biotehniške fakultete in sklepa seje Komisije za doktorski študij Univerze v Ljubljani (po pooblastilu seje Senata Univerze z dne 20. 1. 2009) z dne 6. 7. 2011 je bilo potrjeno, da kandidatka izpolnjuje pogoje za neposreden prehod na doktorski podiplomski študij bioloških in biotehnoloških znanosti ter opravljanje doktorata znanosti na znanstvenem področju genetika. Za mentorico je bila imenovana doc. dr. Bojana Žegura.

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Alja Štraser

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AI Preučevali smo genotoksično delovanje predstavnikov najpogostejših cianotoksinov v sladkih in brakičnih vodah, mikrocistina-LR (MCLR), nodularina (NOD) in cilindrospremopsina (CYN), s posebnim poudarkom na najslabše preučenem CYN. V eksperimentih smo uporabili nizke, za okolje relevantne koncentracije, ki niso citotoksične za celice. Ugotovili smo, da vsi trije cianotoksini povzročajo poškodbe DNK, vendar sta v nasprotju s CYN, NOD in MCLR posredno genotoksična agensa, ki delujeta preko sprožitve oksidativnega stresa. CYN je povzročil obsežne prelome DNK in povišal nastajanje mikrojeder (MNI), jedrnih brstov (NBUD) in nukleoplazmatskih mostičkov (NBP) pri celicah HepG2 in primarnih humanih preifernih limfocitih (HPBL). Poleg tega je po podaljšanem času izpostavitve (72 h) povzročil nastanek dvoverižnih prelomov DNK (DSB). Rezultati transkriptomske analize kažejo, da je CYN pri celicah HepG2 sprožil takojšnji-zgodnji odziv (geni iz družine *FOS* in *JUN*) in signalne poti P53 in NF-κB. Po dolgotrajnejši izpostavitvi je CYN znižal živost celic HepG2, kar pa ni bila posledica celične smrti, temveč znižane celične proliferacije. CYN tudi ni sprožil apoptoze. Analiza celičnega cikla je pokazala, da je CYN povzročil ustavitev celičnega cikla v G0/G1 fazi po 24 h, po daljšem času (72 in 96 h) pa v S fazi, s čimer se skladajo tudi rezultati transkriptomske analize, ki dodatno nakazujejo sprožitev popravljalnih mehanizmov DNK. CYN se je srednje intenzivno presnavljal v prisotnosti mikrosomov in rezultati transkriptomske analize podajajo dokaze za vpletenost številnih metaboličnih encimov I. in II. faze detoksifikacije ksenobiotikov. Rezultati doktorske disertacije kažejo, da so vsi trije cianotoksini genotoksični, vendar CYN predstavlja največje tveganje za zdravje ljudi, saj najverjetneje povzroča poškodbe DNK neposredno in ne povzroča apoptoze pri genotoksičnih koncentracijah, kar potencira tveganje za nastanek raka, še posebej pri dolgoročni izpostavljenosti nizkim koncentracijam.

KEY WORDS DOCUMENTATION

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AB The genotoxic effects of representative cyanotoxins most commonly found in fresh and brackish waters, microcystin-LR (MCLR), nodularin (NOD) and cylindrospremopsin (CYN), with special emphasis on the least studied CYN, were investigated. Low, environmentally relevant, non-cytotoxic concentrations were used in the experiments. We found that all three cyanotoxins cause DNA damage, but in contrast to CYN, are NOD and MCLR indirectly genotoxic agents, acting through the induction of oxidative stress. CYN caused extensive DNA breaks, and increased formation of micronuclei (MNI), nuclear buds (NBUD) and nucleoplasmic bridges (NBP) in HepG2 cells and human peripheral blood lmphoctes (HPBLs). In addition, formation of DNA double strand breaks (DSBs) was detected after prolonged exposure (72 h). The transcriptomic analysis indicated that CYN triggers the immediate-early response (genes from the *FOS* and *JUN* families) and induces P53 and NF-κB signalling in HepG2 cells. After prolonged exposure CYN caused decrease in cell viability of HepG2 cells, which was not due to cell death, but reduced cellular proliferation. In addition CYN did not cause apoptosis. Analysis of the cell cycle showed that CYN induced cell cycle arrest in G0/G1 phase after 24 h and in S phase after prolonged exposure (72 and 96 h), which is in line with the transcriptomic data, that in addition indicates induction of DNA repair mechanisms. CYN is moderately metabolized in the presence of microsomes and the transcriptomic data indicates involvement of a number of phase I. and II. detoxification enzymes. Our results show, that all three cyanotoxins are genotoxic, but CYN poses the greatest threat to human health, as it could cause DNA damage directly and it does not induce apoptosis at genotoxic concentrations, which intensifies the risk of cancer, especially in long-term exposure to low levels.

KAZALO VSEBINE

KAZALO ZNANSTVENIH DEL

OBJAVLJENA ZNANSTVENA DELA

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PREGLEDNI ZNANSTVENI ČLANEK

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KAZALO PREGLEDNIC

KAZALO SLIK

KAZALO PRILOG

KRATICE IN OKRAJŠAVE

4-NQNO	4-nitrokinolin-N-oksid
BaP	benzo[a]piren
BER	popravljanje DNK poškodb z izrezovanjem baz
CDK	od ciklina odvisna kinaza
CDKI	inhibitor od ciklina odvisnih kinaz
CYN	cilindrospermopsin
CYP450	citokrom p450
DNK	deoksiribonukleinska kislina
DSB	dvoverižni prelomi DNK
DSBR	popravljanje dvoverižnih prelomov DNK
ER	endoplazemski retikulum
Fpg	formamidopirimidin glikozilaza
HPBL	primarni humani limfociti iz periferne krvi
HPLC	tekočinska kromatografija visoke ločljivosti
HRR	popravljanje s homologno rekombinacijo
IARC	mednarodna agencija za raziskave raka
Ki67	protein, kazalec celične proliferacije
LC-MS/MS	tekočinska kromatografija s tandemsko masno spektrometrijo
LDH	laktat dehidrogenaza
MCLR	mikrocistin-LR
MHP	Mitohondijska hiperpolarizacija

MMP	mitohondijski membranski potencial
MMR	popravljanje neujemanja v DNK
MNi	mikrojedra
NBUD	jedrni brsti
NER	popravljanje DNK poškodb z izrezovanjem nukleotidov
NHEJ	popravljanje DNK z nehomologninm združevanjem koncev
NOD	nodularin
NPB	nukleoplazmatski mostički
PI	propidijev iodid
QPCR	kvantitativna verižna reakcija s polimerazo v realnem času
ROS	reaktivne kisikove zvrsti
S9	mikrosomalna frakcija jeter

1 PREDSTAVITEV PROBLEMATIKE IN HIPOTEZE

Z vse močnejšo evtrofikacijo voda in s klimatskimi spremembami se vse pogosteje pojavljajo masovna cvetenja cianobakterij. V razmerah pribitka hranil cianobakterijska populacija tvori sekundarne metabolite, številne različne biološko aktivne snovi. Nekatere imajo škodljiv učinek na ljudi in živali, zato jih označujemo kot cianobakterijske strupe oziroma toksine (cianotoksine). Glede na glavno tarčo njihovega delovanja jih v grobem uvrščamo med hepatotoksine, nevrotoksine in dermatotoksine (Carmichael, 2001). V sladkih in brakičnih vodah po vsem svetu se najpogosteje in v najvišjih koncentracijah pojavljajo hepatotoksini mikrocistini (MCs), nodularin (NOD) in cilindrospermopsin (CYN). Ti tokisni lahko povzročajo zastrupitve pri ljudeh, ki se lahko kažejo s simptomi, kot sta slabost in bruhanje, ter bolezenska stanja, ki segajo od vnetja jeter (hepatoenteritis), želodca in prebavil (gastroenteritis) do pljučnice (Falconer s sod., 1983; Hawkins s sod., 1985; Carmichael, 2001). Znani so številni primeri zastrupitev in poginov živali, medtem ko je dobro opisanih primerov zastrupitve ljudi s cianotoksini relativno malo. Razlog za to je verjetno v tem, da ljudje v večini primerov pridejo v stik z nizkimi koncentracijami teh toksinov, ki ne povzročijo dovolj opaznih simptomov. Prav nizke (neakutne) koncentracije in dolgotrajna izpostavljenost pa predstavljajo potencialno nevarnost za ljudi in živali, saj znanstveniki predvsem v zadnjem desetletju ugotavljajo, da cianotoksini lahko poškodujejo DNK (delujejo genotoksično) in morda tudi vplivajo na nastanek rakavih obolenj.

Cvet potencialno toksičnih cianobakterij se dokaj pogosto pojavlja tudi v Sloveniji, predvsem v njenem SV delu, kjer je močno razvito poljedelstvo (Sedmak in Kosi, 1997). V Sloveniji je bilo do sedaj najdenih vsaj 12 potencialno toksičnih vrst cianobakterij (Sedmak s sod., 1994; Sedmak in Kosi, 1997). Najpogosteje se pojavljata predvsem vrsti *Microcystis aeruginosa* in *Plantothrix rubescens* (Eleršek in Kosi, 2010; Bricelj s sod., 2012), ki sta znana proizvajalca mikrocistinov (Fastner s sod., 1999). Vsako leto opažamo prisotnost različnih rodov cianobakterij, ki so potencialni proizvajalci različnih cianotoksinov ter s kemijskimi analizami (HPLC) zaznamo prisotnost mikrocistinov v površinskih vodah, med drugim tudi v Blejskem jezeru (Bricelj s sod., 2012). Kljub temu se razen v kopalnih vodah, ki so pod nadzorom Agencije Republike Slovenije za okolje (ARSO), rasti cianobakterij in koncentracij toksinov v slovenskih vodah ne spremlja.

Raziskav na področju genetske toksikologije cianotoksinov je relativno malo in ugotovitve teh raziskav ne podajo jasnih zaključkov o tem, ali ti toksini povzročajo poškodbe DNK neposredno in ali delujejo tudi rakotvorno (karcinogeno). Raziskave so osredotočene predvsem na preučevanje mikrocistina-LR (MCLR), medtem ko je zelo malo znanega o delovanju drugih cianotoksinov.

Namen raziskav, izvedenih v sklopu doktorske disertacije, je ugotoviti potencialno genotoksične učinke izbranih cianobakterijskih toksinov (mikrocistin-LR (MCLR), NOD in CYN) pri subtoksičnih koncentracijah, s posebnim poudarkom na najslabše raziskanemu CYN, ter ugotoviti mehanizme njihovega genotoksičnega delovanja na modelih humanih celic *in vitro*.

Večino raziskav smo opravili na celicah humanega hepatoma HepG2 (Priloga A), saj so jetra glavni tarčni organ vseh treh izbranih cianotoksinov. Prednost celične linije HepG2 je v tem, da je človeškega izvora, zelo dobro okarakterizirana in je v primerjavi z večino drugih celičnih linij, ki se uporabljajo v *in vitro* raziskavah genotoksičnosti, ohranila določeno mero metabolne aktivnosti (Knasmüller s sod., 2004). Kot model netarčnega organa smo uporabili primarne humane limfocite iz periferne krvi (HPBL) (Priloga B), ki se pogosto uporabljajo v študijah genotoksičnosti. Z različnimi eksperimentalnimi pristopi smo ugotavljali, ali izbrani cianotoksini delujejo genotoksično, ter preučevali celični odziv na izpostavljenost CYN in njegovo presnovo z metaboličnimi encimi.

Glede na problematiko zastavljene doktorske disertacije smo postavili naslednje hipoteze:

- Predvidevamo, da vsi trije izbrani cianotoksini že pri necitotoksičnih koncentracijah povzročajo poškodbe DNK in delujejo genotoksično.

- Glede na predhodne raziskave predvidevamo, da je glavni mehanizem genotoksičnosti MCLR in NOD sprožitev oksidativnega stresa, v nasprotju z CYN, ki predvidoma ne povzroča oksidativnih poškodb DNK.

- Raziskave kažejo, da je CYN pro-genotoksičen. Predvidevamo, da se presnavlja z metaboličnimi encimi iz družine citokrom P450 (CYP450).

- Predvidevamo, da CYN pri izpostavljenih celicah povzroči apoptozo zaradi obsežnih poškodb DNK.

- Predpostavili smo tudi, da CYN zaradi genotoksičnega delovanja in zaviranja sinteze proteinov vpliva na celični cikel.

1.1 PREGLED OBJAV

Genotoxicity and potential carcinogenicity of cyanobacterial toxins - a review

Bojana Žegura, **Alja Štraser**, Metka Filipič

Mutation Research/Reviews in Mutation Research, 2011, 727, 1-2: 16-41.

Zaradi vse močnejše evtrofikacije voda se je bistveno povečalo pojavljanje cianobakterijskih cvetenj po vsem svetu. Cvetenje je nevarno za ljudi, živali in rastline, saj cianobakterije proizvajajo strupe (cianotoksine), ki jih lahko uvrstimo v pet različnih skupin: hepatotoksini, nevrotoksini, citotoksini, dermatotoksini in dražilne spojine. Nekateri cianotoksini so genotoksični in potencialno karcinogeni, vendar pa mehanizmi njihovega delovanja niso dobro poznani. Najpogostejši cianotoksini v brakičnih in sladkih vodah so ciklični heptapeptidi – mikrocistini (MCs) in pentapeptidi – nodularini (NODs). Glavni mehanizem njihovega delovanja je zaviranje proteinskih fosfataz, ki lahko povzroči hiperfosforilacijo celičnih proteinov, in je najverjetneje povezano z njihovo tumor spodbujajočo aktivnostjo. Poleg tega povzročajo tvorbo reaktivnih kisikovih zvrsti in s tem oksidativne poškodbe DNK, povzročajo nastanek mikrojeder in zavirajo delovanje DNK popravljalnih mehanizmov, kar je zelo pomemben dejavnik karcinogenosti. Ti toksini povečajo izražanje TNF-α in genov takojšnjega-zgodnjega odziva, vključno s proto-onkogeni in geni vključenimi v odziv na poškodbe DNK, ustavitev celičnega cikla in apoptozo. MCs in NOD so promotorji tumorske rasti, NOD pa bi lahko celo sprožil nastanek raka. V zadnjem času se v sladkovodnih okoljih vse pogosteje opaža prisotnost cianotoksina CYN. Glavni mehanizem njegovega toksičnega delovanja je ireverzibilno zaviranje sinteze proteinov. CYN je pro-genotoksičen in za aktivacijo potrebuje metabolične encime iz družine citokrom P450. V metabolično kompetentnih celicah povzroča poškodbe DNK in deluje klastogeno in aneugeno. CYN poveča izražanje s P53 reguliranih genov vključenih v ustavitev celičnega cikla, DNK popravljalne mehanizme in apoptozo. Preliminarne študije na glodavcih kažejo, da bi lahko deloval karcinogeno. V letu 2010 je Mednarodna agencija za raziskave raka (IARC) razvrstila MCLR med možne karcinogene snovi za ljudi (skupina 2B), medtem ko za razvrstitev ostalih cianobakterijskih toksinov ni dovolj podatkov. V okolju se ti toksini pojavljajo v kompleksnih zmeseh, skupaj z drugimi antropogenimi onesnaževalci. Številne študije so pokazale, da so ekstrakti cianobakterijskih cvetov bolj toksični in/ali genotoksični od izoliranih toksinov. To pomeni, da lahko te mešanice predstavljajo večje tveganje za zdravje. Zato se moramo v prihodnosti osredotočiti na raziskave karcinogenega potenciala NOD, CYN in skupnega učinka snovi v cianobakterijskih ekstraktih ter identifikacijo morebitnih novih toksinov.

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Review

Genotoxicity and potential carcinogenicity of cyanobacterial toxins – a review☆

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ABSTRACT

The occurrence of cyanobacterial blooms has increased significantly in many regions of the world in the last century due to water eutrophication. These blooms are hazardous to humans, animals, and plants due to the production of cyanotoxins, which can be classified in five different groups: hepatotoxins, neurotoxins, cytotoxins, dermatotoxins, and irritant toxins (lipopolysaccharides). There is evidence that certain cyanobacterial toxins are genotoxic and carcinogenic; however, the mechanisms of their potential carcinogenicity are not well understood. The most frequently occurring and widespread cyanotoxins in brackish and freshwater blooms are the cyclic heptapeptides, i.e., microcystins (MCs), and the pentapeptides, i.e., nodularins (NODs). The main mechanism associated with potential carcinogenic activity of MCs and NOD is the inhibition of protein phosphatases, which leads to the hyperphosphorylation of cellular proteins, which is considered to be associated with their tumor-promoting activity. Apart from this, MCs and NOD induce increased formation of reactive oxygen species and, consequently, oxidative DNA damage. There is also evidence that MCs and NOD induce micronuclei, and NOD was shown to have aneugenic activity. Both cyanotoxins interfere with DNA damage repair pathways, which, along with DNA damage, is an important factor involved in the carcinogenicity of these agents. Furthermore, these toxins increase the expression of TNF-α and early-response genes, including proto-oncogenes, genes involved in the response to DNA damage, cell cycle arrest, and apoptosis. Rodent studies indicate that MCs and NOD are tumor promotors, whereas NOD is thought to have also tumor-initiating activity. Another cyanobacterial toxin, cylindrospermopsin (CYN), which has been neglected for a long time, is lately being increasingly found in the freshwater environment. The principal mechanism of its toxicity is the irreversible inhibition of protein synthesis. It is pro-genotoxic, and metabolic activation by cytochrome P-450 enzymes is needed for its genotoxic activity. In metabolically competent cells, it induces DNA strand breaks and exerts clastogenic and aneugenic activity. In addition, CYN increased the expression of p53 regulated genes involved in cell cycle arrest, DNA damage repair, and apoptosis. It also has cell transforming potential, and limited preliminary rodent studies indicate that CYN could have tumor-initiating activity. In 2010, the International Agency for Research on Cancer (IARC) classified MCLR as possible human carcinogen (Group 2B). Although there is not enough available information for the classification of other cyanobacterial toxins, the existing data from *in vitro* and *in vivo* studies indicate that NOD and especially CYN may be even more hazardous than MCLR to human and animal health. In addition in the environment, cyanobacterial toxins occur in complex mixtures as well as together with other anthropogenic contaminants, and numerous studies showed that the toxic/genotoxic potential of the extracts from cyanobacterial scums is higher than that of purified toxins. This means that the mixtures of toxins to which humans are exposed may pose higher health risks than estimated from the

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Abbreviations: ACF, aberrant crypt foci; AFB1, aflatoxin-B1; ATX, anatoxin-a; ATX(S), anatoxin-a(S); AOM, azoxymethane; BGAS, blue-green algae supplements; bw, body weight; CBE, cyanobacterial bloom extract containing MCs; CDK, cyclin-dependent kinase; CYN, cylindrospermopsin; CYP, cytochrome P; DEN, diethylnitrosamine; DFO, deferoxamine mesylate; DMSO, dimethylsulphoxide; DMTU, 1,3-dimethyl-2-thiourea; DNA-PK, DNA-dependent protein kinase; DSBs, double strand breaks; dw, dry weight; endoIII, endonuclease III; FISH, fluorescence in situ hybridization; fpg, formamydopyrymidine DNA glycosylase; GJIC, gap-junctional intracellular communication; GSH, reduced glutathione; GST-P, glutathione S-transferase placental form; HPLC, high-performance liquid chromatography; IARC, International Agency for Cancer Research; i.p, intraperitoneally; LD_{50}, 50% leathal dose; MAPKs, mitogen-activated protein kinases; MCs, microcystins; MCE, microcystin containing extract; MCLR, microcystin-LR; MCYR, microcystin-YR; MCRR, microcystin-RR; MF, mutation frequency; MNi, micronuclei; MW, molecular weight; neoSTX, neosaxitoxin; NER, nucleotide excision repair; NHEJ, non-homologous end joining; NODs, nodularins; NOD, nodularin; OAT, Porganic anion transporting polypeptides; OECD, Organisation for Economic Co-operation and Development; PCE, polychromatic erythrocytes; PP1 and PP2, protein serine/threonine phosphatases 1 and 2; ROS, reactive oxygen species; STX, saxitoxin; TEMPOL, 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl; TGF-β1, transforming growth factor β1; TNF-α, tumor necrosis factor α; TPA, O-tetradecanoylphorbol 13-acetate; UDS, unscheduled DNA synthesis; WHO, World Health Organization; 8-OH-Gua, 8-hydroxyguanine; 8-oxo-dG, 8-oxo-7,8-dihydro-2′-deoxyguanosine.

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toxicological data of a single toxin. Future research efforts should focus on the elucidation of the carcinogenic potential of NOD, CYN, and the mixture of cyanobacterial extracts, as well as on the identification of possible novel toxins.

Contents

1. Introduction: occurrence and human exposure

The occurrences of cyanobacterial blooms in aquatic environments are increasing in many regions of the world with progressive eutrophication of water bodies and climate change. This is of increasing concern as cyanobacteria from over 40 genera [1] are known to produce a diverse range of toxins (cyanotoxins) (Table 1). Toxic cyanobacterial blooms can be found in eutrophic to hypereutrophic lakes, ponds and rivers throughout the world and are responsible for illness and death of wild and domestic animals [1,2].

The mechanisms behind the toxic effects of cyanotoxins are different because they are structurally diverse chemicals. Defined by their chemical structure, cyanotoxins fall into three groups: cyclic peptides (microcystin and nodularin), alkaloids (anatoxin-a, anatoxin-a(s), saxitoxin, cylindrospermopsin, aplysiatoxin, lyngbiatoxin-a) and lipopolysaccharides [3]. In this review we discuss genotoxic and potential carcinogenic properties of freshwater cyanotoxins, particularly microcystin-LR (MCLR), nodularin (NOD) and cylindrospermopsin (CYN), because these are among the most frequently occurring cyanotoxins in surface and drinking water and are also the most studied regarding the mechanisms of their potential carcinogenicity. The mechanisms of acute toxicity, immunotoxicity and biochemical as well as morphological effects of cyanotoxins are not considered in this review because these topics are covered in depth elsewhere [4–9].

In humans, intoxication with cyanobacteria contaminated water causes symptoms such as nausea/vomiting, weakness, skin irritation, and illnesses ranging from gastroenteritis and pneumonia to hepatoenteritis [1,10,11]. The most severe human intoxication happened in 1996 in Brazil, where 100 of 131 dialysis patients developed acute liver failure due to cyanobacterial contamination of the water used for dialysis and 52 of the patients died [12–14]. The parenteral route of human exposure to cyanotoxins, although a rare event, represents an extremely relevant route with respect to the risk evaluation for human health because it considerably increases the internal dose of the toxins, directly entering the blood-stream [15].

Humans may be exposed to cyanotoxins through several routes. Besides the above-mentioned accidental parenteral route, dermal and inhalation exposure may occur with recreational (sports), professional (i.e., fishing) or domestic (i.e., showering) use of contaminated water [15]. By far the most important, with regard to the frequency of human exposure, is the oral route, which occurs most frequently through the ingestion of contaminated drinking water or accidental swallowing of water during recreational activities. Worldwide, particularly in rural and less-developed countries, where people need to use untreated surface water as the source of drinking water, they are exposed to cell-bound and dissolved cyanotoxins, suffering acute and chronic effect, depending on the amounts of the toxins in the water [16]. Harvested cyanobacterial blooms are used also as organic fertilizer, and it has been shown that microcystins are persistent enough that in certain cases they leach and cause groundwater contamination [17], representing another source of drinking water contamination. Although in humans acute intoxication with cyanotoxins is not very likely, they are of high human health concern due to their potential long-term adverse effects at chronic exposure to low, environmen-

Table 1
Cyanotoxins, found in brackish and freshwater, and their producers. Adapted from: [9,16,164].

Cyanotoxin	Cyanotoxin producing species or genus
Microcystins (MCs)	*Microcystis aeruginosa*, *Microcystis wesenbergii*, *Microcystis viridis*, *Anabaena flos-aquae*, *Oscillatoria agardhii*, *Oscillatoria rubescens*, *Oscillatoria tenuis*, *Haphalosiphon hibernicus*, *Aphanocapsa cumulus*, *Cyanobium bacillare*, *Arthrospira fusiformis*, *Limnothrix redekei*, *Phormidium formosum*, *Hapalosiphon hibernicus*, *Nostoc* sp., *Anabaenopsis* sp., and *Synechocystis* sp.
Nodularin (NOD)	*Nodularia spumigena*
Cylindrospermopsin (CYN)	*Cylindrospermopsis raciborskii*, *Aphanizomenon flos-aquae*, *Aphanizomenon ovalisporum*, *Anabaena lapponica*, *Umezakia natans*, *Raphidiopsis curvata*, *Lyngbia wollei*
Anatoxin-a (Antx-a)	*Anabaena planctonica*, *Anabaena flos-aquae*, *Anabaena spiroides*, *Anabaena circinalis*, *Microcystis sp.*, *Anabaena flos-aquae*, *Aphanizomenon issatschenkoi*, *Aphanizomenon gracile*, *Cylindrospermum* sp., *Raphidiopsis mediterranea* and *Planktothrix spp.*
Homoanatoxin-a (homoAntx-a)	*Oscillatoria formosa*, *Phormidium formosum*, an unknown *Anabaena* sp. and *Raphidiopsis mediterranea*
Anatoxin a-(s) (Antx-a(s))	*Anabaena flos-aquae* and *Anabaena lemmermannii*
Saxitoxins (STX)	*Anabaena flos-aquae*, *Anabaena circinalis*, *Lyngbya wollei* and *Cylindrospermopsis raciborskii*
LPS endotoxins	All cyanobacteria
Microviridin J	*Microcystis* spp

tally relevant concentrations. Epidemiological studies have suggested that cyanotoxins are one of the risk factors for the high incidence of primary liver [18] and colon cancer [19] in certain areas of China, where people consume pond–ditch water contaminated with cyanobacteria. However, the validity of this study is low due to numerous confounding factors (water contaminated also with other chemicals and microbiological agents, and the concomitant exposure to aflatoxin-B1 (AFB1), a known hepatic carcinogen) [15]. Similarly, no conclusions could be drawn from two ecological studies in Florida about possible association between hepatic and colorectal tumors and consumption of drinking water potentially contaminated with cyanotoxins [20].

Dietary exposure to cyanobacterial toxins is also possible; however current information is rather limited. Cyanotoxins can accumulate in crustaceans, shellfish and fish, therefore exposure through consumption of contaminated shellfish or fish may occur [9,21–24]. Human health risk from exposure to toxins produced by harmful algal blooms through the consumption of contaminated sea food is very well known from the marine environment, but this risk posed from freshwater cyanotoxins remains to be assessed [21]. Only few data are available on the accumulation of cyanotoxins in livestock. No MCs were detected in milk of cattle that consumed sub-lethal doses of *Microcystis aeruginosa* [25], while MCs accumulated in the liver, but not in the blood plasma, of cattle fed with *M. aeruginosa* in their drinking water [26]. The World Health Organization in Guidelines [8] concluded that, these concentrations would not present an unacceptable risk to human health. There is evidence that microcystins (MCs) could be transmitted to cultivate plants from contaminated irrigating water [27–29]. Nevertheless, there is low level of concern posed by cyanotoxin exposure via vegetable consumption, as high levels of MCs inhibit plant growth [28]. At low levels, typically found in natural surface waters, the toxins are found only in the roots, however at the levels that is of human health concern (>1 μg/kg) [30]. Another potential source of human exposure, that should not be neglected, is through dietary supplements sold as blue-green algae supplements (BGAS) [31]. These supplements are very popular in the Western countries due to their presumable beneficial effects on human health (weight loss, increasing alertness and energy, anti-depressive and treatment of attention deficit hyperactivity disorders in children [15,32]) [33]. They are mostly produced from the cyanobacteria *Spirulina* spp. and *Aphanizomenon flos-aquae*, which are either grown in artificial ponds or are directly collected from the natural environment [15], where other cyanobacteria contaminants cannot be fully excluded [34]. BGAS are not considered as drugs so there is no prescription or indication for daily dosing and there is no control for cyanotoxin contamination. Recently, it has been shown, that BGAS could contain variable microcystin-LR (MCLR) levels [31,34] and although the cause–effect evidence has not been definitely established, the death of a 34-year-old woman due to liver failure has been suggested to be the consequence of chronic consumption of MCs-contaminated BGAS [35]. BGAS used by the patient contained 2.62–4.06 μg MCLR equivalents/g dry weight (dw), and MCs-positive immunostaining was observed in the patient's liver section [35]. Contamination of BGAS with cyanotoxins is a serious cause for concern, particularly for children, and should not be disregarded or simply underestimated. The health authorities should systematically monitor the situation and, if necessary, establish specific legal limits and quality control of BGAS products [15].

2. Microcystins and nodularins

Globally, the most frequently occurring and widespread cyanotoxins in brackish and freshwater blooms are the cyclic heptapeptides – microcystins (MCs) and pentapeptides, – nodularins (NODs). MCs are produced by several cyanobacterial species (Table 1), whereas NODs are so far known to be produced only by cyanobacteria from the genus *Nodularia* [36]. The biosynthesis of these toxins is nonribosomal, conducted by large multienzyme complexes that include peptide synthetases, polyketide synthases and tailoring enzymes [37,38]. In actively growing cyanobacteria, most of MCs and NODs are located within the cell; very little extracellular toxins are produced. The concentrations of dissolved cyanotoxins in water samples have been detected within concentration range 0.1–10 μg/l [39]. Cell-bound concentrations are several orders of magnitude higher, thus the concentration of dissolved toxins may be much higher in ageing or declining blooms when cell lysis is triggered and the toxins released into environment. Although MCs are chemically stable, in water bodies their microbial degradation can be rapid. However, lag phases can be observed before the degradation occurs, probably because bacteria that can degrade MCs are not always present in sufficient numbers or need to adapt [40]. It has been reported that in the environment MCs can sometimes persist for relatively long time, ranging from 1 to 3 months [41], to up to 6 months in dry scums [42].

MCs are the largest and most diverse group of cyanotoxins, consisting of over 80 congeners [43], whereas only six different NODs have been isolated from *Nodularia* strains [36]. One additional NOD, motuporin, was isolated from a sponge, but is likely the product of a microbial symbiont [44]. MCs share a general structure, which is cyclo(-D-Ala-X-D-MeAsp-Z-Adda-D-Glu-Mdha), where X and Z are variable L-amino acids, d-MeAsp is D-*erythro*-β-methylaspartic acid, Mdha is *N*-methyldehydroalanine. Adda is an unusual C20 amino acid ((2S, 3S, 8S, 9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-diene acid) [39], which is found only in cyanobacterial peptides and is thought to be responsible for the bioactivity of these compounds. The best studied congener among the MCs is MCLR (Fig. 1), characterized by

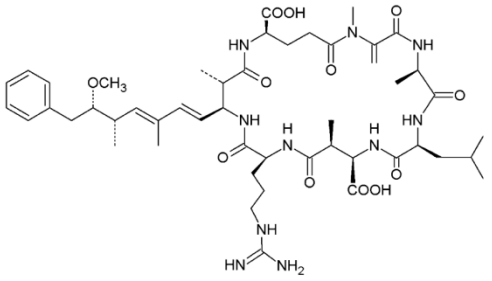

Fig. 1. Structure of microcystin-LR (MCLR).

the presence of leucine (L) and arginine (R) as the variable amino acids [45]. Based on acute toxicity studies MCLR (MW = 995.2) is considered as one of the most potent cyanobacterial toxins. NODs are similar in structure to MCs, and share a structure of, cyclo-(D-MetAsp-Z-Adda-D-Glu-Mdhb), where Mdhb is N-methyldehydrobutyric acid and Z is variable L-amino acid. NOD (Fig. 2), in which Z represented by L-arginine seems to be the main component of environmental samples, with other variants occurring rarely and in negligible concentrations [46,47].

MCs are hydrophilic, they passively penetrate vertebrate cell membranes poorly, and therefore require uptake via active transport. The multi-specific transport system for bile acids has been described as the carrier of MCs in rat liver [48,49]. That is why the liver is their main target and why these toxins have been traditionally considered as hepatotoxins. MCs are now known to be transported through cell membranes by organic anion transporting polypeptides (OATP) [50]. These transporters are not only expressed in the liver, but also in the gastrointestinal tract, kidney, brain, and there is evidence that MCLR can be transported across the human blood–brain barrier [47–50]. The main structural difference between MCLR and NOD is the smaller ring structure of NOD. Therefore, it was proposed that NOD is taken into the hepatocytes more easily than MCLR, which could result in greater cellular effects [51].

MCLR and NOD are specific inhibitors of eukaryotic protein serine/threonine phosphatases 1 and 2A (PP1 and PP2A) *in vitro* [52–55] and *in vivo* [56], with similar potency [57,58]. MCLR binds covalently to protein phosphatases while NOD does not, but this difference does not affect their inhibitory activity [58]. The consequence of protein phosphatase inhibition by MCLR and NOD is hyperphosphorylation of cytoskeletal proteins and thereby disruption of many cellular processes, alteration and rearrangement of the cytoskeleton, loss of cell–cell adhesion at the desmosomes, and consequently disruption of hepatic architecture [49,52,59–65]. More recently it has been shown that also induction of oxidative stress is involved in the hepatotoxicity of MCs [66] and NOD [67,68].

Despite their close resemblance in structure and basic mechanisms of cytotoxicity, several dissimilarities were observed between genotoxic and potential carcinogenic effects caused by MCLR and, NOD *in vitro* [69–71] and *in vivo* [51,70], suggesting different toxicodynamics and different liver accumulation of these toxins [71].

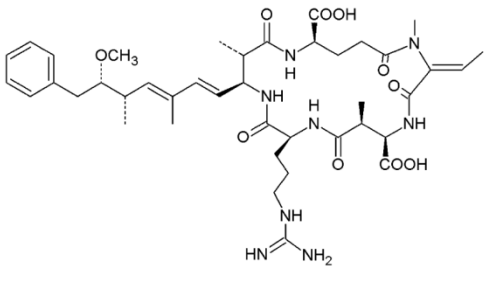

Fig. 2. Structure of nodularin (NOD).

2.1. Genotoxicity and carcinogenicity of MCs

In 1998 the World Health Organization (WHO) established a provisional guideline value for MCLR of 1 μg/l in drinking water that is based on tolerated daily intake (TDI) 0.04 μg/kg body weight [8]. Recently, the International Agency for Research on Cancer (IARC) classified MCLR as possible human carcinogen (Group 2B); whereas, there is not enough available information for the classification of other MCs [5].

MCs are considered to act predominantly as tumor promotors via inhibition of protein phosphatases 1 and 2A, which has been shown to be directly related to their cytotoxicity and tumor-promoting activity [60,72–74]. In the last decade evidence is accumulating showing that MCs induce damage to the DNA (Table 3), which means that they are genotoxic and can act as tumor initiators. However the mechanisms of their genotoxic activity are not clear.

2.1.1. Genotoxicity of MCs in vitro

2.1.1.1. Evidence from bacterial test systems. Genotoxic properties of cyanobacterial extracts and pure toxins have been studied with a variety of bacterial test systems (Table 2). One of the first studies on mutagenic activity of cyanobacterial bloom samples collected in 1978 from Missouri reservoir showed their mutagenicity towards *Salmonella typhimurium* TA1537 [75]. In another study a strong mutagenic response induced by microcystic cyanobacterial extract from a field sample (1.25–125 μg lyophilized algae cells/ml) was demonstrated in four *S. typhimurium* (TA97, TA98, TA100 and TA102) tester strains with and without S9 metabolic activation; however, no mutagenic response was induced by pure MCLR (2.5 μg/ml) [76]. Mankiewicz et al. [77] and Palus et al. [78] reported induction of SOS response by extracts from a field sample from the Sulejów water reservoir, Poland, and pure MCLR (1–10 μM) with and without metabolic activation in *Escherichia coli* PQ37 SOS chromotest system. The extracts with higher content of MCs including MCLR showed higher toxicity and genotoxicity. Pure MCLR was less toxic and genotoxic than the extracts, which indicates the possibility of action of other variants of MCs in the extracts (determined by HPLC), the presence of other genotoxic contaminants [77] as well as synergism among the components of the extract.

Contrary to these results several studies reported negative results in bacterial test systems. *Microcystis* extracts, cyanobacterial bloom extracts, water samples containing cyanotoxins isolated from field samples and pure MCLR did not induce reverse mutation in *S. typhimurium* strains [79–83]. Negative results with cyanobacterial extract isolated from field samples were obtained in the *Bacillus subtilis* multigene sporulation test in both the 168 (excision repair-proficient) and hcr-9 (excision repair-deficient) strains [80] and in the SOS/*umuC* assay with *S. typhimurium* TA1535/pSK1002 [78,83]. In 1995, Tsuji et al. [82] investigated the mutagenic capacity of pure MCLR and its UV-decomposition products. They detected no mutagenic activity of MCLR (50 μg/plate) in Ames assay *S. typhimurium* TA98 and TA100 strains in the presence and absence of metabolic activation S9. Lack of MCLR (20 μg/plate) mutagenic activity was confirmed in another study using *S. typhimurium* TA98 and TA100 strains without S9 mixture [81].

Current evidence indicates that MCs are probably not bacterial mutagens, whereas the discrepancies in the ability of different cyanobacterial extracts to induce mutations in bacteria can be most probably ascribed to different composition of different

Table 2
Genotoxic effects of MCLR and MCE in bacterial test systems.

Test organism/system	Minimum effective MC concentrations	Outcome	Reference
In vitro bacterial assays			
S. typhimurium TA1537	MCE; n.d.	Induction of point mutations	[75]
S. typhimurium TA97, TA98, TA100 and TA102 (with and without S9 metabolic activation)	MCE; 12.5 μg lyophilized cells/ml	No induction of point mutations	[76]
S. typhimurium strains TA98 and TA100	*Microcystis* extracts (4 mg/ml)	No induction of point mutations	[79]
S. typhimurium strains TA98, TA100 and TA102	MCE, MCLR 0.9 mg/ml	No induction of point mutations	[80]
S. typhimurium strains TA98 and TA100 (with and without S9 metabolic activation)	MCLR (50 μg/plate)	No induction of point mutations	[82]
S. typhimurium strains TA98 and TA100 (without S9 metabolic activation)	MCLR (20 μg/plate)	No induction of point mutations	[81,82]
S. typhimurium strains TA98 and TA100	MCE (0.1–100 μg/ml)	No induction of point mutations	[78,83]
S. typhimurium TA1535/pSK1002	MCE (0.1–100 μg/ml)	No induction of genotoxicity	[78,83]
E. coli PQ37 (with and without S9 metabolic activation)	MCE and MCLR (1–10 μM)	Induction of genotoxic response	[77,78]
B. subtilis 168 (excision repair-proficient) and hcr-9 (excision repair-deficient) strains	MCE, MCLR 0.9 mg/ml	No mutagenic activity	[80]

MCE = microcystis cyanobacteria extract; n.d. = not determined; MCLR = microcystin-LR.

extracts regarding the occurrence and ratios of single cyanotoxins in the complex extract mixture. Not only is the composition of cyanotoxins in the mixture important, but also the source of the cyanobacteria, (pure laboratory culture or samples of cyanobacteria collected from the environment). Most of cyanobacterial extracts tested in bacterial test systems were prepared from field samples; therefore, it cannot be excluded that other environmental contaminants were present that could contribute to the genotoxicity of the whole extract.

2.1.1.2. Evidence from mammalian test systems. Genotoxicity of cyanobacterial extract and MCs has been studied *in vitro* and *in vivo* in different mammalian test systems (Table 3).

2.1.1.2.1. DNA damage and DNA repair. In an early study by Ding et al. [76], the genotoxic potential of microcystic cyanobacteria extract isolated from a field sample (equal to 1.25, 12.5 and 125 μg lyophilized algae cells/ml) and pure MCLR (1 μg/ml) was demonstrated on isolated primary rat hepatocytes. The extracts contained MCLR as the main component besides several other MC types and the estimated concentration of MCLR in the microcystis cyanobacteria extract was 0.45 μg/mg lyophilized cells. With the comet assay, which detects single (and double) strand breaks, alkali-labile sites, DNA–DNA/DNA–protein cross-links and single strand breaks associated with incomplete excision repair at the level of single cells [84], a dose dependent increase of DNA damage in cells exposed to microcystis cyanobacteria extract and pure MCLR was observed after 4 h exposure. However, the investigation of morphological changes of hepatocytes treated with the same concentrations of microcystis cyanobacteria extract or MCLLR reviled condensed and segregated nuclei, and most of the cells shrunk, indicating severe cytotoxicity. As dead or dying cells can undergo rapid DNA fragmentation, which increase DNA migration in the comet assay, the observed DNA damage could be the consequence of cytotoxicity rather than genotoxicity. On the other hand, in the same study, *in vivo* in mice the microcystis cyanobacteria extract induced micronuclei formation in polychromatic erythrocytes (PCE), pure MCLR however has not been tested *in vivo*. Later on, the same authors showed that the induction of free radical formation and mitochondrial alterations are associated with MC hepatotoxicity [85]. In human hepatoma cell line (HepG2 cells) MCLR induced DNA strand breaks after exposure to 0.01, 0.1 and 1 μg/ml in time and dose dependent manner [86]. The level of DNA strand breaks increased with time of incubation and reached a maximum after 4 h of exposure and then with further exposure gradually declined. Using the DNA repair inhibitors (cytosine beta-D-arabinofuranoside hydrochloride and hydroxyurea) it was shown that MCLR induced DNA strand breaks were predominantly intermediates of the cellular repair of DNA lesions [86].

In non-hepatic cell lines: baby hamster kidney cells (BHK-21) and mouse embryo fibroblast (MEF) cells, exposure to the extract (100 μg/ml) isolated from pure laboratory cultures of *M. aeruginosa* (UTEX 2385) and purified MCLR (1 μg/ml) exhibited severe DNA fragmentation, which was time-dependent with the peak after 3 h exposure [87]. Recently, it was shown that MCLR, at non-cytotoxic concentrations (0.01, 0.1, 1 and 10 μg/ml) induced time and dose dependent increase of DNA damage as well as increased formation of reactive oxygen species (ROS) in human colon adenocarcinoma (CaCo-2 cells), but not in human B-lymphoblastoid cell line (NCNC cells) [88]. In human lymphocytes cyanobacterial extracts collected from the Sulejóv water reservoir, Poland, containing MCs (0.25–1 μM) and pure MCLR (0.25–4 μM) induced DNA damage, with the maximum reached after 12 h incubation [77]. Pure MCLR induced less DNA strand breaks than the extracts [77]. Palus et al. [78] obtained similar results when studying toxic and genotoxic potential of cyanobacterial extracts collected in the same reservoir during the time course of blooming. Higher cytotoxicity and genotoxicity of the extract correlated with higher content of MCs within the cells. In another study in human lymphocytes, MCLR (1, 10 and 25 μg/ml) induced DNA strand breaks which reached their maximum after 18 h exposure [89]. The authors compared the extent of DNA damage examined by the comet assay to the frequency of apoptotic cells determined by the TUNEL method and found a significant positive correlation. Based on these findings, they concluded that the MCLR induced DNA damage observed in the comet assay may be related to the early stages of apoptosis due to cytotoxicity but not genotoxicity [89].

There is strong evidence suggesting that MCLR induces increased formation of ROS, which cause DNA damage both *in vitro* [61,68,70,71,86,90,91] and *in vivo* [70,71]. In the human hepatoma cell line (HepG2 cells), MCLR (0.01, 0.1 and 1 μg/ml) induced the oxidation of pyrimidines and purines [86,90]. The oxidized bases were detected with the modified comet using the bacterial enzymes endonuclease III (endo III) and formamydopyrymidine DNA glycosylase (fpg), which catalyze the excision of oxidized pyrimidines and oxidized purines, respectively. DNA strand breaks and oxidized pyrimidines were repaired within a short time of exposure to MCLR (8 h), while oxidized purines remained un-repaired in the DNA and accumulated [90]. Of the oxidized purine bases, 8-hydroxyguanine (8-OH-Gua) is the most abundant and most extensively studied lesion. 8-OH-Gua adducts in DNA lead to GC → TA transversion mutations, unless repaired prior to DNA replication [92,93]. Therefore, accumulation of

Table 3
Genotoxic effects of MCLR and MCE in mammalian cell systems and *in vivo*.

Test organism/system	Method	Time of exposure	Minimum effective MC concentration	Outcome	Reference
DNA damage *in vitro*					
Baby hamster kidney cell line (BHK-21 cells) and mouse embryo fibroblast (MEF cells) cell line	DNA unwinding, agarose gel electrophoresis	Up to 3 h	100 μg/ml of *M. aeruginosa* extract (UTEX 2385); 1 μg/ml of MCLR	↑ DNA fragmentation	[87]
Isolated primary rat hepatocytes	Comet assay	4 h	MCE (equal to 1.25 μg lyophilized cells/ml) and pure MCLR (1 μg/ml)	↑ Single strand breaks	[76]
Human hepatoma cell line (HepG2 cells)	Comet assay	1–8 h	0.01 μg/ml MCLR	↑ Single strand breaks (max. after 4 h exposure)	[86]
Human colon adenocarcinoma cell line (CaCo-2 cells)	Comet assay	2–8 h	5 μg/ml MCLR	↑ ROS formation ↑ Single strand breaks (max. after 4 h exposure)	[88]
Human B-lymphoblastoid cell line (NCNC cells)	Comet assay	2, 4, 6, 12, 16 h	10 μg/ml MCLR	No induction of single strand breaks	[88]
Human lymphocytes	Comet assay	12 h	MCLR (0.25 μM) and MCE (containing microcystins 0.25–1 μM)	↑ Single strand breaks	[77]
Human lymphocytes	Comet assay	6, 12, 18 and 24 h	1 μg/ml MCLR	↑ Single strand breaks (related to cytotoxicity and not genotoxicity)	[89]
Human lymphocytes	Comet assay	3 and 6 h	1–4 μg/ml MCLR	↑ Single strand breaks	[78]
Oxidative DNA damage					
Isolated primary rat hepatocytes	^{32}P-postlabelling assay, thin layer chromatography and co-chromatography	3, 6 and 24 h	2 ng/ml MCLR	↑ Formation of 8-oxo-dG	[70]
Human hepatoma cell line (HepG2 cells)	Modified comet assay	2, 4 and 6 h	0.01 μg/ml MCLR	↑ Oxidation of pyrimidines and purines	[86]
Human hepatoma cell line (HepG2 cells)	Modified comet assay	4, 8 and 12 h	0.01 μg/ml MCLR	↑ Oxidation of purines ↑ ROS formation	[90]
Human lymphocytes	Modified comet assay	3 and 6 h	1 μg/ml MCLR	↑ Oxidation of pyrimidines and purines	[78]
Clastogenic and mutagenic activity					
Transformed human embryo fibroblasts (RSa cells)	Resistance towards oubain (QuaR test) PCR and differential dot-blot hybridization	6 days	15 μg/ml MCLR 7.5 μg/ml MCLR	↑ QuaR mutations ↑ Base substitution mutations at K-ras kodon 12	[106]
Chinese hamster ovary cell line (CHO-K1 cells)	Chromosome analysis; Analysis of mitotic spindles	14, 18 and 22 h	MCLR, MCE (25, 50, 100 μM)	↑ Abnormal anaphase with defective chromosome separation and polyploid cells; ↑ Aberrant mitotic spindles	[103]
Chinese hamster ovary cell line (CHO-K1 cells)	Micronucleus assay	MCLR (6 h); subsequent exposure to UV (25 J/m^2) irradiation	10 μg/ml MCLR	No micronuclei formation after exposure to MCLR alone ↑ Frequencies of UV-radiation induced micronuclei	[97]
Human lymphoblastoid *TK* cell line	Micronucleus assay TK gene mutation assay PCR-based LOH	24 h	5 μg/ml MCLR	↑ Micronuclei formation ↑ Frequency of thymidine kinase mutants (assocated with LOH)	[105]
Human lymphocytes	Micronucleus assay	n.d.	MCE	↑ Micronuclei formation	[80]
Human lymphocytes	Chromosome aberrations and mitotic index	6, 12, 18, 24 h	25 μg/ml MCLR	No effect on chromosomal aberrations	[89]
Human lymphocytes	Chromosome aberrations and mitotic index	MCLR (3 h); subsequent exposure to gamma (1 Gy) radiation	0.5 μg/ml MCLR	No micronuclei formation after exposure to MCLR alone ↑ Frequencies of irradiation induced chromosomal aberrations	[98]
Human lymphocytes	Micronucleus assay	44 h	MCE (0, 0.25, 0.5, 1.0, and 2.0 mg ext. of freeze-dried cyanobacteria/ml) containing MCLR, MCYR and MCRR	No micronuclei formation	[102]

Table 3 (*Continued*)

Test organism/system	Method	Time of exposure	Minimum effective MC concentration	Outcome	Reference
Isolated primary rat hepatocytes	^{32}P-postlabeling assay	6 and 24 h	2 ng/ml MCLR	↑ Formation of hydrophobic endogenous DNA adducts (I-compounds)	[71]
Human hepatoma cell line (HepG2 cells)	Micronucleus assay	16 h	0.01 μg/ml MCLR	↑ Micronuclei formation	[104]
DNA repair					
Chinese hamster ovary cell line (CHO-K1 cells)	Comet assay	MCLR (6 h); subsequent exposed to UV (25 J/m^2) radiation ↑ the kinetics of DNA repair at 0, 30, 60 and 120 min post-exposure	10 μg/ml MCLR	Impairment of nucleotide excision repair	[97]
Human lymphocytes	Comet assay	MCLR (4 h); subsequent exposed to gamma radiation (2 Gy) ↑ the kinetics of DNA repair at 0, 15, 30 and 60 min post-exposure	1 μg/ml MCLR	Inhibited repair of gamma induced DNA	[89]
Human lymphocytes	Comet assay Immunofluorescence labeling of g-H2AX foci	MCLR (3 h); subsequent exposed to gamma radiation (1–2 Gy)	0.5 μg/ml MCLR	Inhibited repair of gamma induced DNA damage; ↑ Reduction of g-H2AX foci	[98]
Human glioblastoma cell lines MO59J and MO59K	Comet assay	MCLR (2 h) subsequent exposed to gamma radiation (10 Gy)	0.25 μg/ml MCLR	Inhibited repair of gamma induced DNA damage in MO59K cells	[98]
Genotoxicity *in vivo*					
Male Swiss albino mice: liver cells	DNA unwinding (FADU) Agarose gel electrophoresis	2 h	i.p. 21.5 μg/kg bw MCLR	↑ DNA strand breaks ↑ DNA fragmentation (related to cytotoxicity rather than genotoxicity)	[107]
Male Swiss albino mice: liver cells	DNA unwinding (FADU), Agarose gel electrophoresis	Up to 3 h	32.7 mg/kg bw of *M. aeruginosa* UTEX 2385 extract administered i.p.	↑ DNA fragmentation (related to cytotoxicity rather than genotoxicity)	[87]
Female Swiss albino mice	DNA unwinding (FADU), Agarose gel electrophoresis	30 min	i.p. LD50 (MCLR, MCRR and MCYR at a dose 43 μ, 235.4 and 110.6 μg/kg bw, respectively)	↑ DNA fragmentation	[108]
Female Swiss Albino mice (blood, liver, kidney, ileum and colon, bone marrow)	Comet assay	3 and 24 h	MCLR Oral: 2 mg/kg bw i.p.: 40–50 μg/kg bw (3 h); 10–25 μg/kg bw (24 h)	↑ DNA strand breaks	[111]
Male Kunming mice (polychromatic erythrocytes)	Micronucleus assay	24 h	i.p. MCE (equal to 1 mg lyophilized algae cells/kg bw); twice	↑ Micronuclei formation	[76]
Male CBA mice	Micronucleus assay	42 h	i.p. MCE (equal to 2.0 mg lyophilized algae cells/mouse) or MCLR (0, 10, 20, 30, 34, 38, 42, 46, 50, and 55 μg/kg bw)	No induction of micronuclei	[102]
Male muta ™Mouse (λ/*lacZ* transgenic mice); liver and lung	Mutation assay Micronucleus assay	4 weeks 48 h	MCLR (1 mg/kg bw/week)	No increase in mutant frequency No induction of micronuclei	[114]
Peripheral blood cells					
Male Fischer 344 rats (liver, kidney medulla and cortex, lung and brain, spleen and lymphocytes)	Comet assay	Every second day for 30 days	i.p. 10 μg MCYR/kg bw	↑ DNA strand breaks; No increase of DNA strand breaks in spleen and lymphocytes	[107]
Male Fischer 344 rats	The unscheduled DNA synthesis (UDS) The comet assays	2–4 h 12–16 h	i.v. 50 μg MCLR/kg bw	No incorporation of [3H] thymidine No DNA damage	[112]
Male Sprague–Dawley rats	^{32}P-postlabelling assay, thin layer chromatography, co-chromatography	24 and 48 h	50 μg MCLR/kg bw	↑ Formation of 8-oxo-dG	[70]

MCLR = microcystin-LR; MCE = microcystis cyanobacteria extract; 8-oxo-dG = 8-oxo-7, 8-dihydro-2′-deoxyguanosine; LOH = Loss of heterozygosity; i.p. = intraperitoneal; i.v. = intravenous; bw = body weight; ↑ = effect increase; ↓ = effect decrease; n.d. = not determined.

oxidized purines in cells may lead to mutations. The formation of DNA strand breaks and oxidized purines was prevented by several ROS scavengers [4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPOL), deferoxamine mesylate (DFO), dimethylsulphoxide (DMSO), 1,3-dimethyl-2-thiourea (DMTU)], which provides additional evidence that the genotoxicity of MCLR was mediated by the induction of ROS [90]. The mechanism of MCLR mediated induction of ROS is not clear, but it has been shown that in HepG2 cells oxidative DNA damage was associated with MCLR induced modulation of intracellular reduced glutathione (GSH) content [68,91], which plays an important role in the protection of cellular constituents against ROS [94].

Exposure of primary cultured rat hepatocytes to non-cytotoxic concentrations of MCLR (2 and 10 ng/ml) for 3, 6 and 24 h induced formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG) [70], a common biomarker of oxidative DNA damage. The peak level of 8-oxo-dG adducts was observed after 6 h of exposure and then it declined indicating repair of oxidative DNA damage. The formation of 8-oxo-dG was the result of increased formation of ROS and decreased level of intracellular antioxidants, which has been shown previously by Bouaïcha and Maatouk [68]. The same authors [71] also showed that MCLR did not induce hydrophobic DNA adducts and, thus, did not bind covalently to DNA. Nevertheless, the authors reported that at both tested concentrations MCLR significantly decreased the amount of endogenously formed DNA adducts, termed I-compounds after long-time exposure (6 and 24 h) [71]. I-compounds can play a role in cell transformation and may contribute to carcinogenesis. They are presumably normal DNA modifications, and have been found to be decreased in cancer and other pathological conditions [95]. The decrease of I-compounds in rat hepatocytes was previously reported for several tumor promoters, a non-mutagenic carcinogen (2,3,7,8-tetrachloro-*p*-dibenzodioxin) and a mutagenic carcinogen (2-acetylaminofluorene) [96]. The authors suggested that the depletion of endogenous DNA adducts (I-compounds) and/or the increase of 8-oxo-dG levels by MCLR could be involved in the formation of hepatic tumors during long-term exposure to these cyanobacterial hepatotoxins [71].

Lankoff et al. [97] were the first who showed that MCLR attenuates the nucleotide excision repair (NER). They pre-treated CHO-K1 (Chinese hamster ovary) cells with MCLR (1, 10 and 20 μg/ml) for 6 h, subsequently exposed to UV (25 J/m^2) radiation, and assessed the DNA repair kinetic with the alkaline comet assay after 0, 30, 60 and 120 min of recovery. They confirmed that MCLR alone did not induce DNA damage. In cells treated with 10 and 20 μg/ml MCLR prior to UV-irradiation, the level of residual DNA damage was after 120 min of recovery markedly higher as compared to cells exposed to UV alone. These results suggested that MCLR inhibited the incision/excision phase as well as the rejoining phase of NER [97].

The same authors showed that MCLR inhibited repair (NER) of ionizing radiation induced DNA strand breaks in human lymphocytes, and explored the underlying mechanism of this inhibition [98]. They proposed that MCLR as an inhibitor of protein phosphatases may affect activity of DNA-dependent protein kinase (DNA-PK) that is the key enzyme involved in the non-homologous end joining (NHEJ) of radiation induced DNA strand breaks. In human lymphocytes in G0-phase of the cell cycle, that were pre-treated with MCLR (0.5 μg/ml) for 3 h before the irradiation with 2 Gy of gamma rays, they observed inhibition of the repair of irradiation induced DNA strand breaks, that correlated with the higher frequency of chromosomal aberrations including dicentric chromosomes compared to cells exposed to irradiation alone. In MCLR pre-treated cells compared to the cells that were only irradiated they also found reduced frequency of phosphorylated H2AX (γ-H2AX) nuclear foci. The impact of MCLR on DNA-PK was then confirmed by examining the differences in the kinetics of DNA repair in radiosensitive MO59J (deprived of a catalytic subunit of DNA-PK) and normal MO59K human glioblastoma cells. A strong DNA repair inhibitory effect was observed in the MO59K but not in the MO59J cells, which indicates that DNA-PK could be the target of MCLR [98].

From these studies we can conclude that one pathway of MCs genotoxic activity is mediated by induction of ROS formation, which causes the formation of DNA strand breaks and mutagenic oxidative DNA lesions. In addition, MCLR has been shown to inhibit two pathways of DNA repair, NER and the DSB repair by NHEJ, which is in line with other experiments that demonstrated that PP1 and PP2A inhibition significantly decreases the activity of the DNA repair systems [99,100]. The impairment of DNA repair is along with DNA damage a very important factor involved in carcinogenesis. If the repair process is compromised or the lesion is repaired by some other error-prone mechanism, damaged DNA can cause mutation and ultimately contributes to neoplastic transformation [101].

2.1.1.2.2. Clastogenic and mutagenic activity of MCs. In a very early study, Repavich et al. [80] showed that purified toxin, isolated from field samples, induced a dose dependent clastogenic effect in human lymphocytes. On the contrary Lankoff et al. [89] reported that exposure of human lymphocytes to pure MCLR (1, 10 and 25 μg/ml) for 6, 12, 18 and 24 h had no effect on the frequency of chromosome aberrations, but induced DNA strand breaks and apoptosis (see section DNA damage and DNA repair).

In human lymphocytes exposed to cyanobacterial extracts (0.25, 0.5, 1.0, and 2.0 mg extract of field sample of freeze-dried cyanobacteria/ml) containing MCLR, MCYR and MCRR for 44 h, no increase in the frequency of micro-nucleated cells was detected [102].

Lack of clastogenic activity of MCLR has been shown also in CHO-K1 cells exposed to pure MCLR isolated from pure laboratory culture of *M. aeruginosa* PCC 7813 (25, 50, and 100 μM) [97] and cyanobacterial extract isolated from field samples containing three MCs (MCLR, MCRR and MCYR) for 14, 18 and 22 h [97,103]. However, the toxins led to an accumulation of abnormal G2/M figures with hypercondensed chromosomes in metaphase-arrested cells and a dose dependent increase in the frequencies of abnormal anaphase with defective chromosome separation and polyploid cells [103]. While MCLR per se did not induce MNi in CHO-K1 cells it markedly increased the frequency of UV-induced MNi, which was associated with MCLR mediated diminished efficiency of NER [97]. Similarly, pre-treatment of human lymphocytes with MCLR (0.5 μg/ml; 3 h) inhibited the repairs of radiation induced damage (1 Gy of gamma radiation) and lead to enhanced frequencies of chromosomal aberrations including dicentric chromosomes [98].

Using the micronucleus assay it was shown that MCLR (0, 0.01, 0.1, and 1 μg/ml) induced slight though significant dose dependent increase of MNi formation in HepG2 cells after 16 h of exposure, which shows that MCLR can be clastogenic [104].

Zhan et al. [105] studied genotoxicity of MCLR (5–80 μg/ml) in human lymphoblastoid cell line TK6. In a prolonged 24 h treatment, it induced increase in MNi formation and mutation frequency at the heterozygous thymidine kinase (TK) locus. The molecular analysis of the TK mutants revealed that MCLR specifically induced loss of heterozygosity at the TK locus, but not point mutations or other small structural changes. These results provide further evidence that MCLR has clastogenic activity.

So far only one study reported mutagenicity of MCLR in mammalian cells. In transformed human embryo fibroblasts (RSa cells), exposure to MCLR for 6 days (7.5–15 μg/ml) induced a dose dependent increase in the ouabain resistance (QuaR) mutation frequency and base substitution mutations at K-*ras* codon [106].

From the *in vitro* genotoxicity studies it can be noted that minimal concentrations at which genotoxic effects of cyanobacterial extract or pure MCs have been observed, differ depending on the cell type (Table 3). While in hepatic cells (i.e. isolated primary hepatocytes and HepG2) that presumably express OATP, ROS formation, and DNA damage were observed at nano-molar concentration range, in other cell lines (i.e. peripheral human lymphocytes, lymphoblastoid cell lines, CHO cells...) mikro molar concentrations were required. Mutagenic effects of MCLR have been observed at relatively high, micromolar doses; however, they were tested only in non-hepatic cells. It can be also seen that cyanobacterial extracts and purified MCs in human lymphocytes did not induce MNi formation whereas in HepG2 cells increase in MNi formation was observed at 10 ng/ml. These are important observations that indicate that in target cells the genotoxic effects occur at concentrations that are found in the environment to which humans and animals can be chronically exposed.

2.1.2. Genotoxicity of MCs in vivo

There is a substantial number of *in vivo* studies reporting the genotoxic potential of MCs (Table 3). The fluorometric analysis of DNA unwinding and agarose gel electrophoresis revealed time and dose dependent induction of DNA strand breaks and DNA fragmentation, in liver cells of male Swiss albino mice, treated intraperitoneally (i.p.) with MCLR at 0.5, 1.0 and 2.0 LD_{50} (21.5, 43.0 and 86.0 μg/kg bw, respectively) for up to 2 h [107]. Similar findings were reported in the study with *M. aeruginosa* UTEX 2385 extract isolated from pure laboratory culture (32.7, 65.4 and 130.8 mg/kg bw) that induced significant time and dose dependent DNA fragmentation in liver cells from male Swiss albino mice [87]. In both studies, the authors concluded that the DNA fragmentation was the consequence of the induction of endonucleases and was therefore associated with cytotoxicity rather than direct interaction toxin-DNA. In the same laboratory, the effects of three MC variants were studied in Swiss albino female mice that were i.p. administered with one LD_{50} dose of the toxins (MCLR, MCRR and MCYR at a dose 43, 235.4 and 110.6 μg/kg bw, respectively). Already 30 min after the administration of MCs hepatic DNA fragmentation was observed as an early event occurring before necrotic liver damage [108]. The positive results concerning DNA damage were confirmed in a study where Swiss albino mice (6- and 36-week-old mice) received the LD_{50} dose of MCLR (43.0 μg/kg bw) i.p. or LD_{50} dose of MCLR containing extracts (3.5 g of MCE/kg bw) orally. The increase in DNA damage was age-dependent and the authors suggested that it might be mediated by oxidative stress [109]. The results of a subsequent study revealed that MCLR at 0.5 and 1 LD_{50} (38.31 and 76.62 μg/kg bw, respectively) administered i.p. indeed induced oxidative stress in Swiss albino female mice [110]. Oxidative DNA damage, has been confirmed in a study that demonstrated significant increase of 8-oxo-dG in liver cells from male Sprague–Dawley rats 24 h after i.p. injection of 50 μg/kg bw of MCLR. The 8-oxo-dG levels decreased at 48 h after the MCLR dose but remained significantly higher than in the control [70].

Recently, it was demonstrated that MCLR after single i.p. (25–50 μg/kg bw) administration to female Swiss Albino mice induced DNA damage predominantly in the liver and to lesser extent also in the kidney, intestine and colon, whereas following single oral (2 and 4 mg/kg bw) administration the DNA damage was detected in blood cells, 3 h but not 24 h after the administration. The differences in sensitivity of the i.p. route compared to the oral route suggested a difference in the bio-availability of the toxin [111]. The genotoxic potential of MCLR was studied on male Fisher 344 (F344) rats that were intravenously (i.v.) exposed to the toxin (12.5–50 μg/kg bw). The induction of DNA damage was measured in rat hepatocytes 2–4 h or 12–16 h after the administration with the unscheduled DNA synthesis (UDS) and the comet assays. Neither incorporation of [3H] thymidine nor DNA strand breaks were observed irrespective of time and dose of exposure [112].

In a study on male F344 rats exposed to a sub-lethal dose of purified MCYR isolated from a field sample of *M. aeruginosa* (10 μg/kg bw) by i.p. administration every second day during the period of 30 days, DNA damage was evaluated in several organs using the comet assay. A significant increase of DNA damage in MCYR treated animals compared to the control was detected in the liver, kidney medulla and cortex, lung and brain, while in the spleen and lymphocytes no DNA damage was observed. An interesting finding of this study was the highest level of DNA damage in brain cells [113].

The induction of MNi in polychromatic erythrocytes was evaluated in male Kunming mice that were injected i.p. with cyanobacterial extract isolated from field samples (equal to 1, 10 and 100 mg lyophilized algae cells/kg bw) twice with an interval of 24 h and sacrificed 6 h after the second injection. The authors reported that the frequency of MNi was 5-fold higher in mice exposed to microcystis cyanobacteria extracts (100 mg/kg) compared to control animals [76]. On the contrary, no increase in the frequency of micro-nucleated polychromatic erythrocytes in the peripheral blood of male CBA mice was detected, 44 h after the i.p. injection with a single dose of the cyanobacterial extract isolated from field samples (2.0 mg of lyophilized algal cells/mouse) containing three MCs (MCLR, MCYR and MCRR) or pure MCLR (10, 20, 30, 34, 38, 42, 46, 50, and 55 μg/kg bw) [102].

In λ/*lacZ* transgenic mice (Muta ^{TM}Mouse) treated intragastrically with 0.1 LD_{50} MCLR (1 mg/kg bw/week four times), Zhan et al. [114] demonstrated neither increase of mutation frequencies (MFs) in the *lacZ* and c*II* genes of liver and lungs, nor MNi induction in peripheral blood cells by MCLR.

The results of *in vivo* genotoxicity studies of cyanobacterial extracts and MCLR showed that they readily induced DNA damage in liver as well as in other organs, similar to the *in vitro* studies and indicated that MCs cause oxidative DNA. Induction of micronuclei has been observed only in animals exposed to microcystis cyanobacteria extracts, but not to pure MCs, whereas the study with transgenic mice showed that *in vivo* MCLR is not mutagenic. In the majority of these studies short term exposures to high doses, in the range of LD_{50}, were applied that are far from the doses expected from the environmental exposure. However, the subchronic exposure of rats to sub-lethal dose of MCYR showed DNA damage in multiple organs, including brain, confirming that chronic exposure to MCs results in genotoxic effects.

2.1.3. Carcinogenicity of MCs in animals

In the two-stage carcinogenesis model, the tumor-promoting activity of cyanobacterial extracts and pure MCLR was demonstrated in several animal studies. Although the mechanisms of MCs tumor promotion have not been fully elucidated they most likely involve protein phosphatase inhibition leading to disruption of the dynamic equilibrium of protein phosphorylation/dephosphorylation and hyperphosphorylation of many cellular proteins [115,116] as well as activation of the mitogen-activated protein kinase (MAPK) pathway [117], which may lead to an increase in cellular proliferation [118]. The results of the carcinogenicity studies of microcystis cyanobacteria extracts and MCs are summarized in Table 4.

In a very early tumor promotion study it was shown that a *Microcystis* extract isolated from field samples applied orally to mice caused an increase in the number and size of skin papillomas. Female Swiss albino mice were treated topically on the dorsal epidermis with dimethylbenzanthracene (DMBA) (single dose of 500 μg), and 1 week later they received water containing cell-free extract of *M. aeruginosa* (50 mg MCLR/l) for 52 days. The results revealed that in the DMBA initiated mice exposure to *M. aeruginosa*

Table 4
Potential carcinogenicity of MCLR and MCE in animal studies.

Test organism/system	Method	Time of exposure	Minimum effective MC concentrations	Outcome	Reference
Tumor promotion					
Female Swiss albino mice	Initiated with topical administration of DMBA (500 μg) followed by oral exposure to *M. aeruginosa* extract	52 days	LD50 was 656 mg/kg bw, equivalent to 50 mg MCLR/l	↑ Number and size of skin papillomas	[119]
C57 black mice	Initiated with MNU (two doses of 40 mg/kg) repeated oral administrations of *Microcystis* extract	22 weeks	*Microcystis* extract equivalent to 4.2 MCLR mg/kg/day)	No induction of primary liver tumors No promotion of lymphoid or duodenal tumors	[122]
Male C57Bl/6J mice	Initiated with AOM (i.p. 3 times 5 mg/kg) Followed by repeated oral exposure to *Microcystis* extract after three weeks	31 weeks	382 μg/kg/day of *Microcystis* extract	↑ Size of aberrant crypt foci (ACF) in the colon No changes in the number of crypts per ACFs	[72]
Male Fischer 344 rats	Initiated with DEN (200 mg/kg) followed by partial hepatectomy; repeated i.p. administrations of MCLR	8 weeks	10 μg/kg bw MCLR two times a week	↑ In the number and percentage area of GST-P(+) foci	[73]
Male Fischer 344 rats	Initiated with DEN (200 mg/kg) followed by i.p. admin. of MCLR before (10 μg/kg bw) and after (50 μg/kg bw) partial hepatectomy	8 weeks	10 μg/kg bw MCLR Two times a week	↑ In the number of neoplastic nodules; No induction of liver foci without DEN initiation	[73]
Male Fisher 344 rat	Initiated with DEN without partial hepatectomy; repeated i.p. administrations of MCLR	10 weeks	25 μg/kg bw MCLR twice a week	↑ Induction of GST-P(+) foci	[51]
Fischer 344 rats	Initiated with DEN (200 mg/kg) subsequently Initiated with AFB1 (0.5 mg/kg) followed by repeated i.p. admin. of MCLR after 2 weeks	6 weeks	1 μg/kg bw MCLR two times a week	↑ Number of GST-P(+) foci No synergism between AFB1 and MCLR was observed	[123]
Fischer 344 rats	Initiated with AFB1 (0.5 mg/kg) followed by repeated i.p. admin. of MCLR after 2 weeks	6 weeks	1 μg/kg bw MCLR two times a week	↑ In the number of neoplastic lesions	[123]
Tumor-initiation					
Swiss albino mice	Oral administrations of *M. aeruginosa* extract	54 weeks	*M. aeruginosa* extract (i.p. LD_{50} was 1.7 mg/kg)	Development of bronchogenic and abdominal carcinoma, Thorax lymphosarcoma	[121]
Male Fischer 344 rats	Repeated i.p. administration MCLR without initiator	10 weeks	25 μg/kg bw MCLR two times a week	No induction of liver nodules	[51]
Male Fischer 344 rats	Repeated i.p. administration MCLR without initiator	8 weeks	50 μg/kg bw MCLR two times a week	No induction of liver foci	[73]
ICR mice	Repeated i.p. administration MCLR without initiator	28 weeks	20 μg/kg bw MCLR for 100 times	↑ Induction of liver nodules	[125]

MCLR = microcystin-LR; DEN = diethylnitrosamine; DMBA = dimethylbenzanthracene; AFB1 = aflatoxin-B1; MNU = *N*-methyl-*N*-nitrosurea; AOM = azoxymethane; i.p. = intraperitoneal; GST-P(+) = glutathione S-transferase placental form positive; 8-oxo-dG = 8-oxo-7, 8-dihydro-2′-deoxyguanosine; bw = body weight; ↑ = effect increase; ↓ = effect decrease.

extract induced a nearly 2-fold increase in tumor number and a 7-fold increase in tumor weight compared to animals exposed to DMBA alone, whereas *Microcystis* extract alone did not induce tumors [119,120].

MCs tumorigenesis was investigated in a long-term study, where Swiss albino mice were orally administered *M. aeruginosa* extract isolated from field samples ((1/4, 1/8 and 1/16 dilutions; equivalent to 28.3, 14.1 and 7 μg microcystin/ml; the i.p. LD_{50} of the extract was 1.7 mg/kg bw) for periods up to 1 year. Three mice out of 71 receiving the high concentration of extract (1/4 extract dilution) for 28 weeks developed bronchogenic carcinoma and abdominal carcinoma and one mouse developed thorax lymphosarcoma after 54 weeks. Two out of 73 control animals developed tumors, whereas no tumor developed in 150 mice receiving lower concentrations of the extract (1/8 and 1/16 extract dilution) [121].

To investigate the duodenal and/or adenocarcinoma tumor-promoting activity of MCs, C57 black mice were initiated by *N*-methyl-*N*-nitrosurea (MNU; two doses of 40 mg/kg, p.o.), followed by exposure to a *Microcystis* extract, isolated from field samples (equivalent to 1.2 and 4.2 mg/kg bw/day), in their drinking water for 22 weeks. No primary liver tumors were seen in any group and there was no evidence of extracts induced promotion of lymphoid or duodenal tumors [122].

In another tumor initiation and promotion assay male C57Bl/6J mice were initiated with azoxymethane (AOM), to evaluate possible effects on the colon [72]. Three weeks after the initiation (i.p. 3 times 5 mg/kg AOM), the animals were exposed to *Microcystis* extract, isolated from field samples (equivalet to 382 and 693 μg/kg bw/day, respectively), in their drinking water for 31 weeks. The *Microcystis* extract dose dependently increased the size of individual aberrant crypt foci (ACF) in the colon, while no changes in the number of crypts per ACFs were seen. The observed effect appeared to be a result of an increased rate of cellular proliferation and may indicate tumor progression rather than tumor promotion [72].

In a short term two-stage carcinogenicity bioassay, male F344 rats were i.p. initiated with a single dose (200 mg/kg) of the liver-specific tumor initiator diethylnitrosamine (DEN), followed by partial hepatectomy. The rats were then i.p. administrated MCLR at doses below acute toxicity level (1–50 μg/kg bw), twice a week, during 8 weeks. In DEN initiated rats MCLR induced dose dependent increase in the number and area of glutathione S-transferase placental positive foci (GST-P(+)), which is widely used as a specific marker of carcinogenesis. Administration of MCLR without DEN initiation did not induce liver foci [73]. The tumor-promoting activity of MCLR was confirmed in a similar two-stage carcinogenesis experiment but without partial hepatectomy. The results revealed that MCLR (i.p. 10 and 25 μg/kg bw twice a week from the third week of the experiment) given for 10 weeks stimulated the induction of GST-P(+) foci in male F344 rat liver of DEN initiated rats, while MCLR alone did not induce liver nodules [51].

Sekijima et al. [123] published similar results using AFB1 as a tumor initiator. In the first experiment, DEN initiated (200 mg/kg) rats were given an i.p. injection of AFB1 (0.5 mg/kg) followed by MCLR at 2 weeks after the treatment. In a separate experiment, the rats were first given AFB1 (0.5 mg/kg) and 2 weeks later an i.p. injection of MCLR (1 and 10 μg/kg bw twice a week for 6 weeks). In rats without initiation with DEN, AFB1 induced slight increase in the number of GST-P(+) foci, confirming the hepatocarcinogenic potential of this mycotoxin, while MCLR did not induce GST-P(+) foci. When rats were initiated with DEN both MCLR and AFB1 enhanced the expression of GST-P(+) foci; however, no synergism between AFB1 and MCLR was observed. Results from the second experiment in which the rats were initiated with AFB1 also revealed an enhancement of neoplastic lesions in rats that were subsequently exposed to MCLR.

Ito et al. [124] showed that without an initiator, MCLR (20 μg/kg bw, 100 times for 28 weeks) administered i.p. to ICR mice induced neoplastic nodules up to 5 mm in diameter in the livers of all of the 13 exposed animals, and the liver nodules persisted for a further 2 months after cessation of dosing. According to the authors the liver foci were probably benign tumors. In the same study, no evidence of any liver damage or nodule formation was observed in mice that received MCLR orally by intragastric intubation (80 μg/kg bw, 100 times for 28 weeks) [125]. Although this study indicates that MCLR may act as tumor initiator, the study design (low number of animals) and no data on tumors in control animals, does not allow for firm conclusion.

2.1.4. Carcinogenicity of MCs in humans

Human health problems are most likely related to chronic exposure to low MC concentrations through consumption of contaminated water and food, dermal exposure and inhalation. Epidemiological studies have indicated an association between increased incidence of liver and colon cancer and drinking water sources that were potentially contaminated with MCs in certain areas of China [18,19,126,127]. The incidence of human hepatocellular carcinoma in China is one of the highest in the world and varies geographically [18,128]. There are two proven risk factors that correlate with the increased incidence of the disease; the intake of AFB1 from food items and the hepatitis B virus. The third significant element of association is the source of drinking water [16]. Higher cancer mortality rates are observed in regions where sources of drinking water are ponds and ditches compared to regions where drinking water is drawn from deep wells. As cyanobacteria are abundant in surface waters in south-east China where the incidence of hepatocellular carcinoma is the highest, it has been proposed that MCs in drinking water could contribute to higher incidence of cancer among drinkers of pond and ditch water [16].

2.1.5. The effects of MCs on the expression of proto-oncogene, tumor promoter/suppressor and DNA damage response genes

In addition to the extensive studies of MCLR mediated adverse effects there is also some information on its effect on gene expression (Table 5), which could contribute to a better understanding of the mechanisms of MCLR induced toxicity/genotoxicity and potential carcinogenicity. The first evidence that MCLR modulates the expression of oncogenes and tumor suppressor genes was reported by Sueoka et al. [129]. They showed that in primary rat hepatocytes exposure to MCLR (1 μM) for 6 h remarkably increased the expression of tumor necrosis factor α (TNF-α), which can act as an endogenous tumor promoter [130], and early-response genes from the *jun* and *fos* gene families (*c-jun*, *jun B*, *jun D*, *c-fos* and *fos B*). These genes, also called early-response genes, are proto-oncogenes and the products of these genes are involved in gene regulation in response to a variety of stimuli, including cytokines, growth factors, and bacterial and viral infections.

The influence of MCLR on the expression profile of the proto-oncogenes (*c-fos*, *c- jun* and *c-myc*) was investigated in liver, kidney and testis of male Wistar rats injected intravenously with MC extracts at a dose of 86.7 μg MCLR equivalents/kg bw [131]. Significant induction of these genes at transcriptional and protein level was detected in all organs, with the highest increase observed in the liver. The mRNA levels of the three genes were increased already after 1 h of exposure, and reached a peak at 4 h post-exposure. After 24 h of exposure to the toxin the mRNA levels were again at the control levels. The expression of c-Jun and c-Fos was up-regulated also at the protein level and remained elevated also 24 h after the exposure. c-Jun protein is a positive regulator of proliferation and induces expression of positive regulators of cell

Table 5
Modulation of expression of proto-oncogene, tumor promoter/suppressor and DNA damage response genes following exposure to MCLR.

Gene	Gene product function	Test organism/system	Effective MCLR concentrations	Effect	Observed effect (time)	Reference
p53	Tumor suppressor, regulates the cell cycle, involved in apoptosis, response to DNA damage	Balb/c mice (hepatocytes)	i.p. 50 μg/kg bw MCLR	↑	24 h	[139]
		HepG2 cells	0.01 μg/ml	↑	2 h	[138]
c-jun	Forms the AP-1 (activator protein 1) early-response transcription factor	Male Wistar rat (liver, kidney, testis)	86.7 μg MCLR eq/kg bw	↑	1–24 h; liver, kidney: max at 4 h; testis: max 12 h	[131]
		Primary rat (F433) hepatocytes	1 μM	↑	6 h	[129]
jun-B	Component of the transcription factor complex AP-1	Primary rat (F433) hepatocytes	1 μM	↑	6 h	[129]
jun-D	Functional component of the AP1 transcription factor complex	Primary rat (F433) hepatocytes	1 μM	↑	6 h	[129]
c-fos	Proto-oncogene belonging to the immediate early gene family of transcription factors	Male Wistar rat (liver, kidney, testis)	86.7 μg MCLR eq/kg bw	↑	1–24 h; liver: max at 4 h; kidney, testis: max at 1–2 h	[131]
		Primary rat (F433) hepatocytes	1 μM	↑	6 h	[129]
fos-B	Leucine zipper protein that can dimerize with JUN family proteins, forming the transcription factor complex AP1	Primary rat (F433) hepatocytes	1 μM	↑	6 h	[129]
fra-1	Fos-related antigen 1	Primary rat (F433) hepatocytes	1 μM	0	6 h	[129]
c-myc	Belongs to Myc oncogene family, encodes a transcriptional regulator involved in carcinogenesis	Male Wistar rat (liver, kidney, testis)	Eq MCLR 86.7 μg/kg bw	↑	1–24 h; liver, kidney: max at 4 h; testis: max 12 h	[131]
tnf-α	Endogenous tumor promoter	Primary rat (F433) hepatocytes	1 μM	↑	6 h	[129]
		Female Balb/c mice	i.p. 60 μg/kg bw MCLR	↑	17 h	[136]
p21	Cell cycle arrest	Balb/c mice (hepatocytes)	i.p. 50 μg/kg bw MCLR	↑	24 h	[139]
		HepG2 cells	0.01 μg/ml	↑	2 and 16 h	[138]
		HepG2 cells	0.01 μg/ml	↑	4, 6 and 8 h	
		B6.129-*Trp53*+,+ N5 mice	40 μg/kg bw MCLR	↑	28 days	[140]
gadd45α	Cell cycle G2-M checkpoint, DNA repair process	Balb/c mice (hepatocytes)	i.p. 50 μg/kg bw MCLR	↑	24 h	[139]
		HepG2 cells	0.1 μg/ml	↑	16 h	[138]
		B6.129-*Trp53*+,+ N5 mice	i.p. 40 μg/kg bw MCLR	↑	28 days	[140]
mdm2	Mediates ubiquitination of p53	HepG2 cells	0.01 μg/ml	↑	2, 4, and 6 h	[138]

MCLR = microcystin-LR; i.p. = intraperitoneal; ↑ = increase in gene expression; ↓ = decrease in gene expression; 0 = no induction of gene expression.

cycle progression [132], while c-Fos has oncogenic activity with frequent over expression in tumor cells [133], which may explain tumor-promoting activity of MCLR.

It has been suggested that activation of MAPKs might be an underlying mechanism for the tumor-promoting activity of the serine/threonine protein phophatases inhibitors resulting in increased cellular proliferation [118]. *c-jun* and *c-fos* were reported to be the targets of the MAPK pathway [134]. Once activated MAPK is transferred to the nucleus, where it can activate *c-jun* and *c-fos* gene transcription [4]. In addition, *c-fos* and *c-jun* could be induced via oxidative mechanisms [135]. These findings suggest that induction of the expression of early-response genes might be one possible mechanism for the tumor-promoting activity of MCs.

The expression of TNF-α was investigated in female Balb/c mice exposed to a single i.p. injection of 60 μg/kg bw MCLR. A quantitative RT-PCR revealed a 2.3-fold higher hepatic TNF-α mRNA level in exposed animals compared to the control group 17 h after the administration of the toxin [136]. The level of mRNA expression of TNF-α was also measured in female BALB/c mice i.p. exposed to crude cyanobacteria bloom extracts (CBE) isolated from field samples, containing MCs at four doses of 23, 38, 77 and 115 mg lyophilized cells/kg bw (equivalent to MCs concentration at approximately 7, 12, 24, and 36 μg/kg bw, respectively) for 8 h. The results from the study suggested that high dose of CBE led to the over expression of TNF-α, while low doses significantly inhibited TNF-α gene expression [137].

In a study with HepG2 cells MCLR (0.01, 0.1 and 1 μg/ml) significantly elevated the mRNA expression of tumor suppressor gene *p53* and its downstream-regulated genes involved in DNA repair and cell cycle regulation (the cyclin-dependent kinase (CDK) inhibitor P21WAF1/CIP1 (*P21* gene), the E3 ubiquitin ligase (*MDM2* gene), the growth arrest and DNA damage inducible gene (*GADD45α*) [138]. Elevated expressions of *p53*, *p21* and *gadd45α* genes have also been reported in the study on hepatocytes isolated from MCLR-treated Balb/c mice, which were exposed to different concentrations of MCLR for 24 h. In this study, the mRNA level of more than 60 genes known to be involved in DNA damage response, cell cycle regulation and apoptosis was increased [139]. These data support the assumption that MCs have genotoxic potential.

Recently, Clark et al. [140] identified the key molecular pathways involved in chronic (28 days) and sub-lethal MCLR (40 μg/kg bw) exposure in mice, by using a toxicogenomic approach. Using the microarray technology the increase in the expression of several genes involved in MAPK and janus kinase signaling including janus kinase 1 (*jak1*), *map3k1*, *mapk3*, and *mapk9* was shown. As already mentioned the activation of MAPK leads to an increase in the transcription of genes involved in cellular proliferation including *jun*, *fos*, and *myc*. Indeed, an increase in the expression of the cellular oncogenes *jun*, *jund1*, and *myc* was identified in the study [140]. In addition, several DNA damage responsive genes that are involved in cell cycle regulation (*p21WAF1/CIP1* and *cyclin G*), DNA repair (*gadd45α*) and oxidative stress (*gstm3*) were up-regulated [140].

Toxicogenomic studies showed that *in vitro* and *in vivo* exposure to MCLR induces elevated expression of proto-oncogenes from the *jun*, *fos* and *myc* gene families that are all involved in the stimulation of cell proliferation, and of the tumor necrosis factor α (TNF-α), which explains its tumor-promoting activity. In addition, exposure to MCLR also induces elevated expression of

DNA damage responsive genes that is in line with its observed genotoxic activity.

2.2. Genotoxicity and carcinogenicity of NOD

Like MCs also NOD (MW = 825.0) is suspected to be a human carcinogen; however it has not been studied as extensively as MCs. It is considered to act predominantly as tumor promoter via inhibition of protein phosphatases 1 and 2A; however evidence is accumulating that NOD is also genotoxic. In contrast to MCLR, NOD has been classified by IARC as "animal carcinogen but not classifiable as to its carcinogenicity to humans" (Group 3), predominantly due to the lack of sufficient experimental data [5].

2.2.1. Genotoxicity of NOD in vitro

2.2.1.1. Evidence from bacterial test systems. To our knowledge, no study regarding the mutagenic/genotoxic activity of NOD in bacterial assays has been reported.

2.2.1.2. Evidence from mammalian test systems. Genotoxicity of NOD in mammalian test systems showed that it induced DNA strand breaks, oxidative DNA damage and chromosomal aberrations (Table 6); however, no report is available on its ability to induce gene mutations.

2.2.1.2.1. DNA damage and DNA repair. It has been shown that NOD induces oxidative stress [67] and production of intracellular ROS, which was associated with modulation of intracellular GSH content and lipid peroxidation [68].

In primary cultured rat hepatocytes NOD (2 and 10 ng/ml), similar to MCLR, induced time and dose dependent formation of 8-oxo-dG adducts [70]. In cells exposed to NOD the peak level of 8-oxo-dG adducts was observed after 3 h of exposure, which after 24 h of exposure dropped to the level of the control cells. In MCLR treated cells, the peak level was reached after 6 h of exposure and after 24 h did not decline to control level [70] (see chapter 2.1.1.2). This indicates that NOD and MCLR differ in the kinetics of ROS induction and the kinetics of formation of oxidative DNA damage. Like MCLR also NOD did not form DNA adducts, but caused a decrease in endogenously formed DNA adducts, I-compounds. Compared to MCLR the I-compound decrease in NOD exposed cells was stronger [71].

Induction of oxidative DNA damage by NOD isolated from a field sample (1, 2.5, 5 and 10 μg/ml), was shown in HepG2 cells, using the modified comet assay with fpg enzyme that catalyzes the excision of oxidized purines [141]. The highest level of DNA strand breaks was reached after 12 h exposure, while the oxidized purines reached the peak level after 24 h exposure. Within 48 h exposure DNA strand breaks were completely repaired, while the level of oxidized purines remained elevated. Similar kinetics of induction of oxidized purines was also observed in HepG2 cells exposed to MCLR, except that the MCLR induced oxidative DNA damage was repaired more rapidly (within 8 h of exposure) [90].

Lankoff et al. [142] reported that NOD, isolated from a field sample, interferes with the NER in CHO cells. The wild type cells, which are NER proficient and three mutant variants (XPG$^-$, XPF$^-$ and ERCC1$^-$), which are deficient in the incision step of NER [143], were pre-treated with NOD (1, 10 and 20 μg/ml) for 6 h. Subsequently the cells were irradiated with UV light (20 J/m^2) and the comet assay was preformed after 0, 15, 30 and 60 min of recovery to assess the kinetics of the repair of UV-induced DNA damage. In the wild type cells, that were only UV irradiated, the level of DNA strand breaks increased during the first 30 min and then decreased again, which is in line with the kinetics of the repair of UV-induced DNA damage [144,145]. In UV irradiated mutant cells the formation of strand breaks was significantly lower, which is in line with their deficiency to perform the incision step of NER. In UV irradiated NOD (10 and 20 μg/ml) pre-treated wild type and XPG$^-$ cells, was the level of DNA strand breaks after 15 and 30 min significantly lower than in cells exposed to UV alone. In the XPF$^-$ and ERCC1$^-$ cells, pre-treatment with NOD showed no effect. The authors suggested that NOD suppresses the XPF/ERCC1 complex and that this is likely to be one of the mechanisms responsible for NOD induced genome instability.

NOD exerts effects comparable to those induced by MCLR: it induces ROS formation, oxidative DNA damage, DNA strand breaks and interferes with the NER. Some differences have been observed in the kinetics of oxidative DNA damage formation.

2.2.1.2.2. Clastogenic and aneugenic activity. The clastogenic effects of NOD were investigated in CHO-K1 cells [69] and in HepG2 cells [141] using the micronucleus assay. Increased frequency of MNi

Table 6
Genotoxic effects of NOD.

Test organism/system	Method	Time of exposure	Minimum effective NOD concentrations	Outcome	Reference
In vitro					
Human hepatoma cell line (HepG2 cells)	Comet assay	6 h 12 h	5 μg/ml NOD 2.5 μg/ml NOD	↑ DNA fragmentation	[141]
Human hepatoma cell line (HepG2 cells)	Modified comet assay	6 h 12 h	2.5 μg/ml NOD 1 μg/ml NOD	↑ Oxidized purine formation	[141]
Human hepatoma cell line (HepG2 cells)	FISH micronucleus assay	24 h 48 h	5 μg/ml NOD 2.5 μg/ml NOD	↑ Centromere positive micronuclei	[141]
Primary rat (Sprague–Dawley) hepatocytes	^{32}P-postlabeling of 8-oxo-dG	3 h	2 ng/ml NOD	↑ 8-oxo-dG formation	[70]
Primary rat (Sprague–Dawley) hepatocytes	^{32}P-postlabeling assay	6 h	2 ng/ml NOD	↑ Of hydrophobic endogenous DNA adducts (I-compounds)	[71]
Chinese hamster ovary cell line (CHO cells)	Comet assay	NOD (6 h); subsequent irradiation UV light (20 J/m^2) → the kinetics of DNA repair at 0, 30 and 60 min post-exposure	10 μg/ml NOD	Impairment of nucleotide excision repair ↑ Level of UV-induced cytogenetic DNA damage	[142]
Chinese hamster ovary cell line (CHO-K1 cells)	Micronucleus assay	24 h	10 μM NOD	↑ Micronuclei formation ↑ Polynucleated cells	[69]
In vivo					
Sprague–Dawley rat liver	^{32}P-postlabeling of 8-oxo-dG	48 h	50 μg/kg bw NOD	↑ Formation of 8-oxo-dG	[70]

NOD = nodularin; 8-oxo-dG = 8-oxo-7, 8-dihydro-2′-deoxyguanosine; FISH = fluorescent in situ hybridization; fpg = formamydopyrymidine DNA glycosylase; bw – body weight; ↑ = effect increase; ↓ = effect decrease.

was found in CHO-K1 cells incubated with NOD (10 μM) for 24 h, whereas MCLR induced only a slight effect at 50 μM concentration. In addition, an increase of polynucleated cells, which could be considered a marker for aneugenicity, was observed for NOD, but was not further investigated. In HepG2 cells, no increase in MNi formation was observed after 6 and 12 h while after 24 and 48 h increased frequency of MNi was detected at concentrations >2.5 μg/ml. The analysis of MNi with fluorescence in situ hybridization (FISH) using a centromeric probe revealed that most of the induced MNi were centromere positive, indicating that NOD acts as aneugen. Although there is no direct experimental evidence that aneugenic activity might result from the inhibition of protein phosphatase PP2A by NOD, it is known that PP2A plays a role in maintaining of the functional integrity of the cytoskeleton, as it participates in the modulation of cell differentiation and division [146]. A deregulation or inhibition of PP2A leads to a defective sister chromatide separation, aberrant amplification of centrosomes and the formation of abnormal mitotic spindles [103,147,148]. These results indicated that NOD shows stronger clastogenic activity than MCs, and that it induces aneuploidy. However, when comparing clastogenicity of NOD to that of MCs it should be noted that MCs, so far, have not been studied for their aneugenic activity.

2.2.2. Genotoxity of NOD in vivo (Table 6)

The only *in vivo* study on the potential genotoxic effect of NOD by Maatouk et al. [70] showed that single i.p. administration of NOD (50 μg/kg bw) to male Sprague–Dawley rats induced formation of 8-oxo-dG adducts. A slight increase in 8-oxo-dG adducts was observed 24 h after the injection, while after 48 h the level of 8-oxo-dG adducts was 2.7-fold higher than in the control cells. In the same study MCLR induced the appearance of 8-oxo-dG adducts earlier, which after 48 h of exposure tended to decline (see Section 2.1.2), indicating differences in the kinetics of the induction of the oxidative DNA damage between NOD and MCLR.

2.2.3. Carcinogenicity of NOD in animals

Evidence obtained from experiments with animals exposed to sub-lethal doses of NOD is suggesting NOD to be a carcinogen (Table 7). In a two-stage carcinogenicity bioassay, male F344 rats were initiated with 200 mg/kg DEN without partial hepatectomy [51]. Two weeks after the initiation, the rats were exposed to NOD, isolated from pure laboratory cultures of *Nodularia spumigena* LS75 (10 and 25 μg/kg), through repeated i.p. administrations, twice-weekly, for 10 weeks. In DEN initiated rats, NOD induced a dose dependent increase in the number of GST-P(+) liver foci. In addition, the repeated i.p. administrations of NOD (25 μg/kg) alone induced GST-P(+) foci, indicating that NOD itself had an initiating activity. Compared to MCLR, which has been evaluated in the same study (see Section 2.1.3), NOD had a much stronger tumor-promoting activity, and on the contrary to MCLR exerted also tumor-initiating activity.

NOD tumor promotion activity was confirmed in another two-stage carcinogenicity assay in which the F344 rats were initiated with DEN (200 mg/kg bw i.p.) and 2 weeks later they were i.p. injected with NOD (25 mg/kg bw twice/week) until week 12 [149]. The rats were sacrificed during NOD exposure (at the end of weeks 8 and 10), at cessation of NOD (week 12) and after NOD cessation (end of weeks 15, 18 and 22). In DEN and NOD alone groups of animals, only minimal formation of aberrant GST-P(+) foci was observed. In DEN/NOD groups the maximal number (but not area) of GST-P(+) foci was observed at the week 8, which then declined after cessation of NOD injections. Anti-PCNA (proliferating cell nuclear antigen) immunostaining showed high proliferative index in GST-P(+) foci, and in the background hepatocytes during NOD exposure, which after NOD cessation returned to control level in

Table 7
Potential carcinogenicity of NOD in animal studies.

Test organism/system	Method	Time of exposure	Minimum effective NOD concentrations	Outcome	Reference
Tumor promotion					
Fischer 344 rats	Carcinogenesis initiated with DEN, repeated i.p. administrations of NOD Imunohistochemical staining (anti-GST-P antibodies)	Two times a week 10 weeks	10 μg NOD/kg bw	↑ Number and volume of GST-P(+) foci	[51]
Fischer 344 rats	Carcinogenesis initiated with DEN, repeated i.p. administrations of NOD Imunohistochemical staining (anti-GST-P and anti-PCNA antibodies)	Two times a week 10 weeks	25 μg NOD/kg bw	↑ Number and volume of GST-P(+) foci and nodules Increase in PCNA index	[149]
Tumor-initiation					
Fischer 344 rats	Repeated i.p. administrations of NOD Imunohistochemical staining (anti-GST-P antibodies)	Two times a week 10 weeks	25 μg NOD/kg bw	↑ GST-P(+) foci formation	[51]
Fischer 344 rats	Repeated i.p. administrations of NOD Imunohistochemical staining (anti-GST-P antibodies)	Two times a week 10 weeks	25 μg NOD/kg bw	Transient GST-P(+) foci formation	[149]

NOD = nodularin; DEN = diethylnitrosamine; i.p. = intraperitoneal; GST-P(+) = glutathione S-transferase placental form positive; PCNA = proliferating cell nuclear antigen; bw = body weight; ↑ = effect increase; ↓ = effect decrease.

the background hepatocytes, but not GST-P(+) foci. This study showed that NOD, as tumor promoter, imposes proliferative pressure on initiated hepatocytes that is reflected as the persistent presence of highly proliferating GST-P(+) foci even after its cessation in non-hepatectomized rat liver.

2.2.4. The expression of proto-oncogenes and tumor promoter/supressor genes

In vitro and *in vivo* studies showed that NOD modulates the expression of proto-oncogenes, tumor suppressor and tumor promoter genes (Table 8). Ohta et al. [51] showed 3 and 6 h after the single i.p. administration of NOD (50 μg/kg bw, isolated from pure laboratory cultures of *N. spumigena* L575) to F344 rats, strongly up-regulated mRNA expression of genes from the *jun* and *fos* families of early responsive genes: *c-jun*, *jun-B*, *jun-D*, *c-fos*, *fos-B* and *fra-1* in the liver. The same observation was later confirmed in primary cultured rat hepatocytes treated with 1 μM NOD [129]. In addition, NOD, isolated from *N. spumigena* from field samples, strongly induced expression of TNF-α and its release into the medium. The expression of early responsive genes and TNF-α was prolonged for up to several hours, which was completely different from the reported time course of *c-jun* or *c-fos* genes expression induced by other growth stimuli such as O-tetradecanoylphorbol 13-acetate (TPA) or serum growth factors. Investigations of the mechanisms of prolonged expression of these genes showed that NOD (and also MCLR) stabilized the mRNA, and the authors proposed that it could be due to the inhibition of protein phosphatases 1 and 2A, or the interference with pathways regulated by AU rich elements [129] that are involved in degradation of mRNA [150].

The two-stage carcinogenicity bioassay as described in the previous section (Section 2.2.3) [149] was employed to further investigate molecular mechanisms of NOD induced carcinogenesis [151,152]. To determine the mechanism for selective proliferation of enzyme altered hepatocytes in GST-P(+) nodules, changes in the expression of transforming growth factor β1 (TGF-β1) and TGF-β1 receptors I and II (RI and RII) were analyzed [151]. TGF-β1 is a cytokine, belonging to the growth factor TGF-β family, which is known to regulate a wide variety of cellular processes including cell growth, apoptosis, and differentiation, among others [153]. During the exposure to NOD, the expression of TGF-β1 was strongly elevated in the GST-P(+) nodules, while the expression of RI and RII was lost. After cessation of NOD injections, in a part of the nodules the expression of RII appeared again. These results strongly indicate, that selective proliferation of hepatocytes in GST-P(+) nodules is due to avoidance of TGF-β1 induced hepatocyte regression, through loss of TGF-β1 receptors. In another study the expression of *p53*, which is the most common molecular target involved in human carcinogenesis [154], was analyzed in rat hepatic parenchyma and Kupfer cells [152]. In both types of cells *p53* expression was transiently increased for 3 days after DEN/NOD injections, but the same was also seen after the treatment with DEN alone. Therefore, this effect could be closely associated with the damage and/or regeneration of liver parenchyma and removal of the damaged cells through apoptosis.

2.3. Summary of indication for genotoxicity and carcinogenicity of MCs and NOD

The main mechanism associated with potential carcinogenic activity of MCs and NOD is the inhibition of protein phosphatases, which leads to the hyperphosphorylation of cellular proteins. In contrast to MCLR, NOD does not bind covalently to PP1 or PP2A, while it exerts genotoxic effects comparable to those induced by MCs. Pure MCs are not bacterial mutagens, whereas NOD has not been tested in bacteria. The *in vitro* studies with mammalian cells and *in vivo* rodent studies showed that both toxins induce ROS formation, DNA damage and micronuclei formation. NOD increased the frequency of polynucleated cells as well as centromere positive micronuclei, suggesting that it acts as an aneugen. However, for MCs there is currently no data on their potential aneugenic activity. MCs and NOD interfere with the DNA damage repair pathway, NER, while MCLR inhibits also the DSB repair by NHEJ, which can contribute to genetic instability of the exposed cells. Furthermore, both toxins induce increase in gene expression of TNF-α and early-response genes, including proto-oncogenes that might be one possible mechanism for their tumor-promoting activity. Regarding their carcinogenic activity in animals MCLR and NOD induce the formation of GST-P(+) liver foci in the presence of an initiator, while NOD induces GST-P(+) liver foci also in non-initiated animals. Although NOD has been studied less extensively than MC, the reported data indicate that its genotoxic and carcinogenic potential might be even stronger than that of MCs.

Numerous genotoxicity studies, in particular the early ones have been performed with microcystis cyanobacterial extract from field samples. In addition to induction of DNA damage and tumor promotion, the extracts, but not pure MCs, were also mutagenic in bacteria. In most cases the effects observed after the exposure to cyanobacterial extracts were more pronounced than after the exposure to pure MCs. However, due to the possibility that these extracts, particularly those collected from the environment, contain apart from cyanotoxins also other environmental contaminants, it is impossible to ascribe all the effect to cyanobacterial toxins. Still these results are very valuable as they reflect the real situation to which humans and animals can be exposed.

3. Cylindrospermopsin

Cylindrospermopsin (CYN), a cyanobacterial hepatotoxin, was first identified and isolated from the cyanobacterium *Cylindrospermopsis raciborskii* in 1992 [155]. It is now known that CYN is produced by several other freshwater cyanobacterial species as well (Table 1). CYN is a stable 415 Da alkaloid, containing uracil, which is linked to a tricyclic guanidine group through a hydroxyl bridge (Fig. 3) [155]. The molecule is zwitterionic and highly water soluble, due to the positively charged guanidine group and the negatively charged sulphate group [156]. Only two other natural occurring structural variants, 7-epi-CYN from *Aphanizomenon ovalisporum* [157] and 7-deoxy-CYN from *C. raciborskii*, *Rhapidiopsis curvata* and *Lyngbia wollei* [158–161] have been identified so far. The first variant was reported to have similar toxic potency as CYN [157], whereas the toxicological studies on 7-deoxy-CYN are controversial [162,163]. Nevertheless, both variants need to be considered in the CYN risk assessment process.

CYN is increasingly being found worldwide, and was implicated in human intoxications and animal mortality [11,164,165]. The main target of CYN activity is the liver, but other organs such as the kidneys, lungs, thymus, spleen, adrenal glands, intestinal tract, the immune system and the heart might be affected [11,166–168]. The principal mechanism of CYN toxicity is the irreversible inhibition of protein synthesis [162,166]. There appear to be two toxic responses [169]. The rapid toxicity seems to be mediated by cytochrome P450 (CYP450) generated metabolites [170–173] and the longer-term toxicity is due to protein synthesis inhibition. There is evidence that CYN is more toxic in the short term repeated dosing exposure (1–2 weeks) than in the long-term exposure [174]. Therefore, the acute oral LD_{50} in experimental animals could not be clearly established. Using the standard safety factors for subchronic toxicity in rodents, a provisional guideline value for CYN in drinking water was proposed at 1 μg/l [156,174].

The uracil group was proposed to be essential for CYN toxicity [162,175]. CYN also inhibits glutathione synthesis [170,173],

Table 8
Modulation of expression of proto-oncogenes and tumor promoter/suppressor genes following exposure to NOD.

Gene	Gene product function	Test organism/system	Effective NOD concentrations/time of exposure	Effect	Observed effect (time)	Reference
Tgf-β1	Transforming growth factor, a cytokine, hepatocyte growth inhibitor, apoptosis initiator, proliferation stimulator	Rat (F344) liver	25 μg NOD/kg bw two times a week, 10 weeks	↑	8–12 week of the experiment	[151]
Tgf-β1 RI and RII	Transforming growth factor β receptor (serine/threonine kinase)	Rat (F344) liver	25 μg NOD/kg bw two times a week, 10 weeks	Ø	8–15 weeks of the experiment	[151]
p53	Tumor suppressor, regulates the cell cycle, involved in apoptosis, response to DNA damage	Rat (F344) liver	25 μg NOD/kg bw two times a week, 10 weeks	↑	For 3 days after treatment	[152]
c-jun	Forms the AP-1 (activator protein 1) early-response transcription factor	Rat (F344) liver	50 μg NOD/kg bw	↑	3–9 h	[51]
			3, 6, 9 and 24 h		Max at 3 h	
		Primary rat (F433) hepatocytes	1 μM NOD	↑	Max at 1 h	[129]
			1, 4, 10 and 24 h		1–24 h	
jun-B	Component of the transcription factor complex AP-1	Rat (F344) liver	50 μg NOD/kg bw	↑	3–9 h	[51]
			3, 6, 9 and 24 h		Max at 6 h	
		Primary rat (F433) hepatocytes	0.1 μM NOD	↑	1–24 h	[129]
			1, 4, 10 and 24 h			
jun-D	Functional component of the AP1 transcription factor complex	Rat (F344) liver	50 μg NOD/kg bw	↑	6 h	[51]
			6h			
		Primary rat (F433) hepatocytes	0.1 μM NOD	↑	1–24 h	[129]
			1, 4, 10 and 24 h			
c-fos	Proto-oncogene belonging to the immediate early gene family of transcription factors	Rat (F344) liver	50 μg NOD/kg bw	↑	6 h	[51]
			6 h			
		Primary rat (F433) hepatocytes	0.1 μM NOD	↑	4 h	[129]
			1, 4, 10 and 24 h			
fos-B	Leucine zipper protein that can dimerize with JUN family proteins, forming the transcription factor complex AP-1	Rat (F344) liver	50 μg NOD/kg bw	↑	6 h	[51]
			6 h			
		Primary rat (F433) hepatocytes	0.1 μM NOD	↑	4, 10, 24 h	[129]
			1, 4, 10 and 24 h			
fra-1	Fos-related antigen 1	Rat (F344) liver	50 μg NOD/kg bw	↑	6 h	[51]
			6 h			
		Primary rat (F433) hepatocytes	0.1 μM NOD	↑	4, 10 h	[129]
			1, 4, 10 and 24 h			
tnf-α	Tumor necrosis factor	Endogenous tumor promoter	Primary rat (F433) hepatocytes	0.01 μM NOD	1–24 h	[129]
			1, 4, 10 and 24 h	↑		

NOD = nodularin; bw = body weight; Ø = loss of expression; ↑ = increase in gene expression.

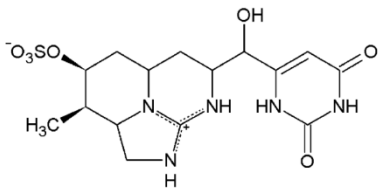

Fig. 3. Structure of cylindrospermopsin (CYN).

which could lead to an increase in oxidative stress that could contribute to its genotoxicity. Nevertheless, oxidative stress and ROS formation were found not to be likely the cause for its genotoxicity, as no changes in the markers for oxidative stress were observed after exposure to CYN [173]. Since CYN has several potential targets for reactivity, protein and DNA adducts could occur with CYN itself or CYP450 generated reactive products from CYN, suggesting the potential for DNA strand cleavage or mutation during DNA replication [169]. Particularly the presence of the uracil group suggests that CYN could be interacting with adenine groups in RNA and DNA, interfering with DNA synthesis and therefore induces mutations and acts as a carcinogen [176].

3.1. Genotoxicity and carcinogenicity of CYN

There has been an indication of CYN carcinogenicity for humans, as increased gastrointestinal cancer incidence was observed years after intoxication with CYN in the exposed population, compared to a similar unexposed population, but the sample size was insufficient for statistical analysis (Falconer, unpublished data). *In vitro* and *in vivo* studies have shown CYN to be genotoxic and several studies imply that it is pro-genotoxic (Table 9). Although preliminary evidence has shown that CYN could have tumor-initiating activity in a range of rat tissues [176], and cell transforming potential of CYN was shown in Syrian hamster embryo (SHE) cells [177], CYN mediated carcinogenesis has not been well evaluated in animal studies. The studies addressing this topic are scarce and the mechanisms of CYN carcinogenicity are not well understood. Therefore, CYN was not evaluated by the WHO, but US EPA has classified it on the list of compounds with high priority for hazard characterization [178].

3.1.1. Genotoxicity of CYN in vitro

3.1.1.1. Evidence from bacterial test systems. To our knowledge, no study regarding the mutagenic/genotoxic activity of CYN in bacterial assays has been reported.

3.1.1.2. Evidence from mammalian test systems.

3.1.1.2.1. DNA damage. Employing the comet assay, CYN, isolated from pure laboratory cultures of *C. raciborskii* Seenayya Subba Rayu, was shown to induce DNA strand breaks in primary mouse hepatocytes [173]. A dose dependent increase in DNA damage was found in cells treated with cytotoxic and non-cytotoxic doses of CYN (0.05–0.5 μM) for 18 h. In order to explore if the DNA damage was a consequence of CYP450-activated CYN metabolites, CYP450 inhibitors were used. Both, the broad-spectrum monooxygenase inhibitor SKF525A (50 μM) and the CYP "3A4/2C19" inhibitor omeprazole (100 μM) completely prevented CYN genotoxicity. In CHO-K1 cells exposed to CYN, isolated from pure laboratory cultures of *C. raciborskii* AWQC CYP-026J (0,5 and 1 μg/ml), for 24 h, no induction of DNA strand breaks was detected [179]. As no exogenous metabolic activation was used in this study, the reason for the lack of DNA damage can be ascribed to low metabolizing enzyme activity in CHO-K1.

3.1.1.2.2. Clastogenic and aneugenic activity. No chromosome aberrations were detected in CHO-K1 cells after the exposure to CYN isolated from pure laboratory cultures of *C. raciborskii* AWT 205 and CYN-Thai (0.05–2 μg/ml), for 3, 16 and 21 h regardless the activation with S9 fraction (post mitochondrial supernatant prepared from rat liver-S9 mix) [180]. These results do not exclude that CYN metabolites are involved in its genotoxicity. The negative results could have been due to lack of an appropriate metabolic system, since some CYP forms (e.g. CYP1B1 or CYP2E1) are low or inactivated in standard S9 fraction [181]. In addition, the external metabolic activation can result in different cell effects, compared to effects caused by CYN metabolites that are formed within the cell, due to longer diffusion pathways that give more opportunities for alternative chemical reactions. In addition, competing pathways, leading to inactive metabolites, may be more prevalent with S9 than with more specific enzyme systems [181]. Although CYN did not induce DNA damage [179] and chromosomal aberrations [180] in CHO-K1 cells it (1 and 5 μM, 24 h) affected the microtubular structure in this cell line, which could disrupt spindle or centromere function and may lead to loss of whole chromosomes [182].

In contrast to the studies with CHO-K1 cells, it was recently reported, that CYN induced MNi formation in two human cell lines: the liver-derived HepaRG and the colon-derived Caco-2 that are both metabolically active [183]. These two cell lines were used as hepatocyte- and enterocyte-like models, respectively, representing known target organs of CYN. The involvement of CYP metabolism in its genotoxicity was investigated by employing the CYP inhibitor, ketoconazole. In undifferentiated HepaRG cells, there was no increase in MNi formation following exposure to CYN (0.5–2 μg/ml). However, MNi formation was significantly increased in differentiated HepaRG cells treated with low doses of CYN (0.04–0.3 μg/ml) for 24 h. These findings are contributing to the evidence for pro-genotoxicity of CYN, as HepaRG cells express metabolic enzymes at levels comparable to those in primary human hepatocytes in their differentiated state [184,185]. In the undifferentiated state HepaRG have lower CYP expression, except for CYP 1A1 and 7A1 [185]. Moreover, CYN (0.5–2 μg/ml) significantly and dose dependently increased the formation of MNi in undifferentiated Caco-2 cells after 24 h treatment. The same was seen in differentiated Caco-2 cells, but to a lesser extent. Pre-treatment with ketoconazole (10 μM), which inhibits CYP3A4, 1A1, 1A2, 1B1, 2B6, 2C8, 2C9, 2C19, 2D6, 4F2 and F12 [186–188], showed no effect on CYN induced MNi in differentiated HepaRG cells. However, ketoconazole alone increased the level of MNi in differentiated HepaRG cells. In Caco-2 cells, the addition of ketoconazole reduced CYN induced MNi by about 50%. Therefore, it seems that CYN genotoxicity is mediated through its metabolites and that minor CYP isoforms may play role in its metabolic activation.

CYN induced MNi formation was also reported in a human lymphoblastoid cell line, WIL2-NS [168]. CYN isolated from pure laboratory cultures of *C. raciborskii* induced a significant increase of MNi formation in cells exposed to 6 and 10 μg/ml CYN for 24 h. In addition, cells with complete loss of normal nucleus appeared, in which the nuclear material was distributed among a number of MNi. The analysis of the MNi for the presence of centromeres by FISH showed a dose dependant (1–10 μg/ml) increase of centromere positive MNi, indicating aneugenic activity of CYN. The increase in centromeres in micro-nucleated binucleated cells could not fully account for the greater increase in numbers of MNi in binucleated cells, especially as many of the MNi contained multiple centromeres. These results suggest that CYN has both clastogenic and aneugenic activity. This means that it causes structural and numerical chromosomal aberrations that are events known to be implicated in carcinogenesis and heritable diseases. The clasto-

Table 9
Genotoxic and potential carcinogenic effects of CYN.

Test organism/system	Method	Time of exposure	Effective CYN concentrations	Outcome	Reference
***In vitro* genotoxicity**					
Primary rat hepatocytes	Comet assay	18 h	0.05–0.5 μM CYN	↑ DNA damage No DNA damage after inhibition of CYP450	[173]
Chinese hamster ovary cell line (CHO-K1 cells)	Comet assay	24 h	0.5, 1 μg/ml CYN	No DNA damage	[179]
Chinese hamster ovary cell line (CHO-K1 cells	Chromosome aberration assay, with and without metabolic activation (S9)	3, 16, 21 h	0.05–2 μg/ml CYN	No clastogenic activity (No chromosome aberration)	[180]
HepaRG differentiated cells	Micronucleus assay	24 h	0.04–0.3 μg/ml CYN	↑ Micronuclei formation	[183]
Caco-2 undifferentiated and differentiated cells	Micronucleus assay	24 h	0.5–2 μg/ml CYN	↑ Micronuclei formation	[183]
WIL2-NS lymphoblastoid cell line	Micronucleus assay	24 h	6, 10 μg/ml CYN	↑ Micronuclei formation	[168]
WIL2-NS lymphoblastoid cell line	FISH micronucleus assay	24 h	1, 3, 6, 10 μg/ml CYN	↑ Centromere positive micronuclei formation	[168]
***In vivo* genotoxicity**					
Balb/c mice liver	Analysis of DNA strand breakage by alkaline gel electrophoresis	6, 12, 24, 48 h	0.2 mg CYN/kg bw	↑ DNA strand breakage	[189]
White	^{32}P-postlabeling assay	24–96 h	1 μg CYN*/kg bw	↑ DNA adduct formation	[174]
Quackenbrush mice	Autoradiography				
Swiss Albino mice colon	Comet assay	24 h	4 mg CYN/kg bw i.p. 0.1, 0.2 mg/kg bw	↑ DNA damage	[190]
Swiss Albino mice bone marrow	Comet assay	24 h	1, 2 mg/kg bw	↑ DNA damage	[190]
Swiss Albino mice blood, liver, kidney, ileum	Comet assay	24 h	1, 2, 4 mg CYN/kg bw i.p. 0.05 0.1, 0.2 mg/kg bw	No DNA damage	[190]
Swiss Albino mice bone marrow, colon crypts	Micronucleus assay	24 h	1, 2, 4 mg CYN/kg bw i.p. 0.05 0.1, 0.2 mg/kg bw	No micronuclei formation	[190]
***In vitro* carcinogenicity**					
SHE cells	SHE cell transformation assay	7days	10^{-13} to 10^{-8} μg/ml CYN	↑ Cell transformation	[177]
***In vivo* carcinogenicity**					
Swiss Albino mice	Post-mortem examination of organs and histological examination	30 weeks after last dosing	1× 1500 mg CYN/kg bw 3× 500 mg CYN/kg bw	Tumor-initiation	[176]

CYN = cylindrospermopsin; FISH = fluorescent in situ hybridization; bw = body weight; CYP450 = cytochrome P450; ↑ = effect increase.

genic activity is most likely the consequence of DNA strand brakes at higher CYN concentrations that induce formation of MNi with chromosome fragments. The aneugenic activity could arise due to kinetochore or spindle disruption, possibly mediated via protein inhibition, which leads to formation of MNi containing whole chromosomes. However, metabolism of CYN by WIL2-NS cells is yet to be confirmed, therefore the involvement of CYN metabolites is not clear.

3.1.2. Genotoxicity of CYN in vivo

The formation of DNA adducts after exposure to CYN, isolated from pure laboratory cultures of *C. raciborskii* AWT 2 05/1, was detected *in vivo* in Quackenbush mice. The mice were given a single i.p. dose of CYN (0.001 mg/kg) and were periodically killed after 24–96 h [174]. The DNA was extracted from mice liver and adducts were determined using ^{32}P-postlabeling assay and two-dimensional electrophoresis. Although only a single adduct spot was observed in each case, this suggests that covalent binding of CYN or a CYN metabolite to DNA could occur.

CYN, isolated from laboratory cultures of *C. raciborskii*, induced DNA strand breakage was shown *in vivo* in Balb/c mice [189], that were treated with a single dose of CYN (0.2 mg/kg bw; i.p.) and were sacrificed after 6, 12, 24, 48 and 72 h. DNA was isolated from mice liver and the presence of strand brakes was investigated by alkaline gel electrophoresis (pH 12). The results revealed significant increase in DNA strand breaks that was the highest 24 h after the exposure.

In a recently published study, *in vivo* genotoxicity of CYN has been evaluated in male Swiss Albino mice treated with CYN by a single i.p. injection (0.05, 0.1 and 0.2 mg/kg bw) or by gavage (1, 2 and 4 mg/kg bw) [190]. 24 h after the treatment the comet assay was performed on the liver, blood, bone marrow, kidney, intestine and colon samples, and the micronucleus assay on bone marrow and colon cells. In i.p. injected mice, increased DNA damage was observed only in the colon (0.1 and 0.2 mg/kg bw). In orally exposed mice DNA damage was observed in addition to the colon (4 mg/kg bw) also in the bone marrow (1 and 2 mg/kg bw), which is one of the most resistant organs in respect to DNA damage [191]. However, no increase in the MNi formation was observed in the bone marrow and in the colon, irrespective of the route of CYN administration [190]. Because only one sampling time (after 24 h) was used in this study, it is possible that the time was to short to reveal MNi formation in particular because CYN is an inhibitor of protein synthesis which is known to slow down cell cycle progression.

3.1.3. Carcinogenicity of CYN

A preliminary exploration of CYN carcinogenicity was carried out with male Swiss Albino mice [176]. Mice were orally dosed with an extract of cultured *C. raciborskii* AWT 205, containing CYN (5.5 mg/g freeze-dried extract). Five mice, which received the extract at the dose of 1500 mg/kg two times, survived and were retained in the study. Further, 14 mice were treated only once with this dosage and 34 mice received three doses of 500 mg/kg. The individual doses were separated by 2 weeks. Subsequently, a subgroup of the mice received (10 μg) of the tumor promoter TPA, twice weekly for 30 weeks. After that time the mice were euthanized and histological analysis of the major organs revealed 5 animals with tumors among 53 mice treated with CYN extract, while no tumors were found in the control group (27 mice). No effect of the tumor promoter was observed on treated or control mice. Although the number of mice used in this experiment was insufficient to provide statistical significance for tumor initiation by CYN, the calculated relative risk indicates a potential biological and public health threat.

To elucidate the carcinogenic potential of CYN, its cell transforming activity was assessed, using the Syrian hamster embryo (SHE) cell transformation assay [177]. This assay was recommended by the Organisation for Economic Co-operation and Development (OECD) for evaluation of the carcinogenic potential of chemical, physical and biological agents [192]. Cell transforming potential was found at very low CYN doses (10^{-7} to 10^{-2} ng/ml), while higher CYN concentrations did not induce cell transformation, which may be due to different biotransformation patterns at low and high concentrations of CYN. Terao et al. [166] observed a decrease in CYP450 protein in the liver of mice treated with CYN, thus it is possible that at higher concentrations of CYN the synthesis of CYP 450 is reduced and consequently also the formation of CYN metabolites that could be involved in cell transformation, and are formed at low CYN concentrations.

3.1.4. The expression of DNA damage response genes

CYN is also modulating the expression of some DNA damage response genes. Exposure to CYN, isolated from pure laboratory cultures of *C. raciborskii* AWT 205, resulted in elevation of mRNA levels of P53 regulated genes (*CDKN1A*, *GADD*45α, *BAX* and *MDM*2) in P53 proficient cell lines, HepG2 and human dermal fibroblasts (HDF), but not in Caco2 cells [193], which express an inactive form of P53 [194]. After 6 h of treatment with CYN, the expression of all four tested genes increased in HepG2 and HDF cells, suggesting an early activation. Also after 24 h exposure to 1 μg/ml of CYN, relative mRNA levels were elevated compared to control cultures. Despite the induction of P53 regulated genes, no accumulation of P53 protein was detected in HDF or HepG2 cells even at concentrations up to 5 μg/ml. This may be due to a reduction in de novo P53 synthesis arising from CYN mediated translational inhibition, or increased expression of MDM2 and subsequent degradation of P53. Although several other studies indicate pro-genotoxicity of CYN, the authors presume that the elevation of expression of the p53 regulated genes, seen in HepG2 and HDF cells, was due to the native toxin and not its metabolites [193].

3.2. Summary of indication for genotoxicity and carcinogenicity of CYN

It is now generally accepted that CYN is pro-genotoxic and needs to be metabolically activated by cytochrome P-450 enzymes to become genotoxic. In metabolically competent cells it induces DNA strand breaks and MNi formation, showing its clastogenic effect. Furthermore, it increases centromere positive MNi, which suggests that it acts as an aneugen. In addition, CYN increased the expression of p53 regulated genes involved in cell cycle arrest, DNA damage repair and apoptosis. It has cell transforming potential and there is some indication that CYN could have tumor-initiating activity. All these results together show, that CYN is genotoxic and probably more hazardous to human and animal health than MCs. However, due to inadequate evidence, more studies need to be conducted to fully assess its genotoxic and especially carcinogenic potential.

4. Other cyanotoxins

4.1.1. Anatoxin-a and homoanatoxin-a

Anatoxin-a (ATX), cyanobacterial neurotoxin, is a potent post-synaptic depolarizing neuromuscular blocking agent [195]. It is an alkaloid (MW = 165), a bicyclic secondary amine (2-acetyl-9-azabicyclo[4,2,1]non-2-ene) (Fig. 4) [195], produced by several strains of cyanobacteria (Table 1). Indicated by the name, it was first isolated and characterized from the cyanobacterium *Anabaena flos aquae* [196]. A homologue of ATX, which is also a potent neuromuscular blocking agent, is homoanatoxin-a [197]. It was first isolated in 1992 from the cyanobacterium *Oscillatoria formosa*

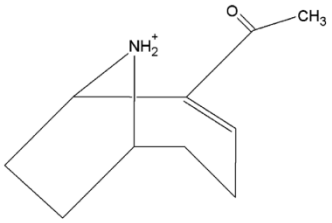

Fig. 4. Structure of anatoxin-a (ATX).

[198]. Instead of the acetyl group in ATX, homoanatoxin-a has a propionyl group, and a molecular weight of 179 g/mol.

ATX is a nicotinic (cholinergic) agonist that binds to neuronal nicotinic acetylcholine receptors at the neuromuscular junction, with grater affinity than the neurotransmitter acetylcholine or nicotine [199]. Since this cyanotoxin is not degraded by the enzyme acetylcholinesterase, the binding causes persistent receptor stimulation and therefore persistent muscle stimulation. A sufficiently high dose can lead to muscular paralysis and death by respiratory arrest within minutes [200]. No known therapy exists for intoxications with this cyanotoxin, other than respiratory support [201].

Although the acute neurotoxic effects of ATX have been studied extensively, there is nearly no information regarding the genotoxicity or carcinogenicity of this alkaloid or its homologue [200]. There is only one study showing cytotoxic activity of ATX in non-neuronal cells and the induction of ROS formation in cultured rat thymocytes [202], which could cause DNA damage. It was proposed that ATX is unlikely to be of significant concern with regard to long-term toxicity [200]. Therefore, the acute neurological effects were suggested to be the main cause for concern when considering human health risk assessment [203]. However, it is important to stress that there are no studies regarding potential genotoxicity of ATX; therefore, we cannot say that long-term toxicity has no importance for human health risk, especially because there are also no data available regarding the ability of ATX to cross the blood–brain or placenta barrier.

4.1.2. Anatoxin-a(s)

Anatoxin-a(S) (ATX(S)) is an organophosphate that acts as a non-competitive irreversible inhibitor of acetylcholinesterase activity in the neuromuscular junctions [204]. ATX(S) is a unique 252 Da guanidinium methyl phosphate ester (Fig. 5), structurally related to organophosphorous insecticides. Its chemical structure is unrelated to ATX, but as it was isolated from the same species (*A. flos aquae*) and demonstrated similar signs of poisoning as ATX, it was termed ATX(S), which is causing confusion [204]. The letter "S" stands for salivation factor and corresponds to the characteristic symptom of salivation caused in vertebrates [1]. Structural variants of ATX(S) have not been detected so far.

As the consequence of inhibition of acetylcholinesterase by ATX(S), acetylcholine is not degraded and remains attached to the membrane receptors, resulting in continuous muscle stimulation and death may occur by respiratory arrest as with ATX [205]. There are few toxicity studies of ATX(S). One of the reasons may be the instability of this toxin, as it becomes inactivated at elevated temperatures (>40 °C) and under alkaline conditions [1]. Furthermore, ATX(S) does not occur as frequently as MCs and its concentrations in scums will scarcely reach levels that are acutely neurotoxic to a human ingesting a mouthful [206].

Repavich et al. [80] studied the genotoxicity and mutagenicity of ATX(S) and neosaxitoxin (neoSTX), isolated from water samples

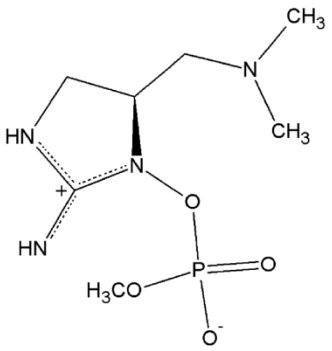

Fig. 5. Structure of anatoxin-a(S) (ATX(S)).

from lakes in Wisconsin. No mutagenic activity of purified lyophilized extracts with ATX(S) or neoSTX has been observed (dose-levels 25–100 μl of a stock with 0.02 mg ATX(S)/ml and a stock with unknown concentration of neoSTX), with the Ames assay (*Salmonella* strains TA98, 100 and 102) with or without metabolic activation [80]. The *Bacilus subtilis* multigene sporulation test using excision repair-proficient 168 and excision repair-deficient hcr-9 strains also showed negative results [80]. On the other hand, both ATX(S) (0.8 μg/ml) and neoSTX (0.005 and 0.0005 ml of a stock with unknown concentration) increased chromosomal breakage in short term cultures of human lymphocytes [80].

4.1.3. Saxitoxins and neosaxitoxin

Saxitoxins (STXs) are a group of carbamate alkaloids that consists of 27 known variants [207], which are either nonsulphated (STX), singly sulphated (gonyautoxins, GTXs) or doubly sulphated (C-toxins) [16]. In addition, decarbamoyl derivatives (dc) and several new toxins (*Lyngbya wollei* toxins, LWTXs) have been identified in some cyanobacterial species [16]. STXs and neosaxitoxins (neoSTXs) possess a unique tricyclic structure with hydropurine rings and guanidine subunits (Fig. 6).

All STXs act in the same way but significantly differ in toxicity, with STX being the most toxic [15]. STXs bind to the outer ion conduction pore of the Na^+ channels in neuronal cells, and so block the nervous transmission [208,209] and cause asphyxiation due to progressive respiratory muscle paralysis. They also block Ca^{2+} and K^+ channels in cardiac cells, thus preventing the propagation of the action potential, which may cause fatal cardiac arrhythmias [210,211].

Originally STXs were isolated from shellfish where they accumulate from marine dinoflagellates [16] but the same variants of STXs are also produced by some species of freshwater cyanobacteria (Table 1). The chemical structure and the toxico-

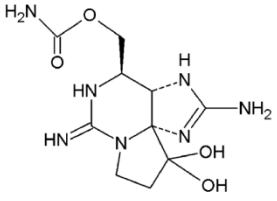

Fig. 6. Structure of saxitoxin (STX).

logical profile of the toxins are the same, independent of their source. The great majority of reported toxicological data have been obtained with STXs produced by marine organisms and only limited information is available for STXs produced by freshwater cyanobacteria. To date no reports of human poisonings due to freshwater STXs are known. There are also no experimental data for subchronic exposure, reproductive, teratogenic or carcinogenic effects of STXs on mammals.

4.1.4. Unknown cyanotoxins and their interactions

Cyanobacteria produce many other bioactive compounds. Several new cyclic or linear peptides and depsipeptides (peptides with an ester linkage) from cyanobacteria have been characterized. Some are protease inhibitors, but the biological activity of the others remains to be characterized [212,213]. Not only that there are probably still numerous unidentified cyanotoxins, cyanobacterial bioactive compounds could have additive, synergistic, potentiating or antagonistic effects. Several studies indicate that cyanobacterial water blooms contain many unidentified components that may evoke toxic effects that could be more pronounced than those of chemically characterized cyanotoxins [214–216]. In addition, it has been shown that the synthesis of non-hepatotoxic cyclic peptides can mediate the release of various toxic and otherwise biologically active substances that can amplify the toxic effects of cyanotoxins [217] and may even induce systemic genotoxicity in mammals [218]. These assumptions are supported by several studies showing that crude cyanobacterial extracts containing MCLR exerted stronger mutagenicity, DNA damage and MNi formation compared to the effects caused by the purified toxin [76–78].

Recently, Bláha et al. [219] reported the tumor promotion potencies of cyanobacterial extracts with unknown bioactive compounds. Using rat liver epithelial stem-like cells (WB-F344) they investigated inhibition of gap-junctional intercellular communication (GJIC) and activation of the MAPKs – ERK1/2. Cyanobacterial extract mediated modulation of GJIC was assessed using a scrape loading-dye transfer assay and activation of ERK1/2 by determination of phosphorilated ERK1/2 by Western blotting. Extracts of cyanobacteria (laboratory cultures of *M. aeruginosa* and *A. flos-aquae* and water blooms dominated by these species) inhibited GJIC and activated MAPKs in a dose dependent manner (effective concentrations ranging 0.5–5 mg dry weight/ml). Effects were independent of the MCs content and the strongest responses were elicited by the extracts of *Aphanizomenon* sp. that did not contain MCs. Due to the presence of *Aphanizomenon* sp., the extract could contain CYN; however the extract was not analyzed for its presence. Neither pure MCLR (100 μg/ml) nor CYN (25 μg/ml) at higher concentration that would be expected in the extracts inhibited GJIC or activated MAPKs. Thus, the strong effects of cyanobacterial extracts on GJIC and ERK1/2 were probably MC and CYN independent, which indicates the existence and presence of unidentified toxins that could potentially contribute to tumor promotion.

5. Summary

Cyanobacterial blooms are characterized by the presence of several cyanobacterial species, usually dominated by one, but also within a single-species, there may be a mixture of non-toxic and toxic strains. Cyanobacteria can produce various toxins from hepatotoxins (microcystins, nodularins), neurotoxins (anatoxins, saxitoxins), cytotoxins (cylindrospermopsin), skin irritants (lipopolysaccharidic endotoxins), gastrointestinal toxins and many yet unknown biologically active metabolites.

Cyanobacterial toxins have become widely recognized as a problem that is arising as a consequence of increased surface water eutrophication. The major route of human exposure to cyanotoxins is oral by using contaminated water for drinking and by incidental ingestion of contaminated water during recreational and professional activities. Lately, several other exposure sources, such as consumption of contaminated freshwater organisms and dietary supplements prepared from blue-green algae (BAGS), are gaining attention. Exposure to high levels of cyanobacterial toxins in drinking and recreational waters have been associated with numerous animal deaths and some cases with human illness, leading to substantial concern for human exposure. The reason that there are few well-substantiated cases of human cyanotoxin exposure is that the levels of exposure are just below those that cause symptoms that people would notice and associate with cyanobacteria contamination. However, the experimental evidence from *in vitro* and *in vivo* studies shows that they are of high concern. Chronic exposure to low concentrations of these toxins may, due to their potential long-term adverse effects (genotoxic, carcinogenic, reproductive), increase the risk for cancer development. It is unlikely that massive transient cyanobacterial blooms are associated with the risk of chronic exposure; however, in regions with persistent cyanobacterial blooms and intensive recreational activities, subchronic exposure may cause adverse health effects.

Often the predominant toxins in freshwater bodies worldwide are MCs that have been implicated in several cases of animal and human intoxications. MCs are hepatotoxic cyclic peptides and are considered to act predominantly as tumor promoters via inhibition of protein phosphatases 1 and 2A. Pure MCs are not mutagenic in bacteria, whereas in mammalian cells they have been shown to induce ROS formation and DNA damage; some studies have also showed micronuclei formation. Current evidence indicates that MCs cause DNA damage indirectly by the induction of reactive oxygen species formation rather than having a direct genotoxic effect. In hepatic cells (i.e., isolated primary hepatocytes and HepG2 cells) that presumably express OATP activity, ROS induction and associated genotoxic effects were observed at nano-molar concentrations that are relevant for environmental contamination and, thus, low-dose chronic exposure. Another important mode of action reported for MCs is their interference with two DNA repair pathways: NER and the DSB repair by NHEJ. Besides DNA damage, the impairment of DNA repair is an important factor involved in the processes of carcinogenesis. If the repair process is compromised or the lesion is repaired by some other error-prone mechanism, damaged DNA can cause mutations, which ultimately could contribute to neoplastic transformation. Thus, by induction of oxidative DNA damage and subsequent inhibition of DNA repair, exposure to low concentrations of MCs could contribute to genomic instability and, consequently, to an increased risk for cancer. These results, together with long-term carcinogenicity studies in rodents, provide the evidence that MCLR should be considered a genotoxic carcinogen and contributed to its classification by IARC as possible human carcinogen (Group 2B) [5].

Another cyanobacterial toxin with hepatotoxic properties, NOD, is currently classified as suspected to be a human carcinogen (IARC Group 3) [5]. The structure and basic mechanisms of its toxicity are similar to those of MCs. Like MCs, NOD is also considered to act predominantly as a tumor promoter via inhibition of protein phosphatases 1 and 2A. In contrast to MCLR, NOD does not bind covalently to PP1 or PP2A. The results from carcinogenicity studies show that NOD is a stronger tumor promoter than MCLR and also has tumor-initiating activity. *In vitro* NOD exerts genotoxic effects comparable to those induced by MCs. It induces ROS formation and, consequently, oxidative DNA damage. In addition, NOD increases the frequency of polynucleated cells as well as centromere positive micronuclei, suggesting that it acts as an aneugen. Similar to MCLR it interferes with the DNA

damage repair pathway, NER, whereas MCLR also inhibits DSB repair by NHEJ. Furthermore, MCLR and NOD modulate the expression of oncogenes and tumor-suppressor genes, with strong induction of tumor necrosis factor-α, and *c-jun*, *jun-B*, *jun-D*, *c-fos*, *fos-B* and *fra-1* gene expression, which might be one possible mechanism for their tumor-promoting activity. In animals both, MCLR and NOD induce the formation of GST-P(+) liver foci in the presence of an initiator; however, NOD induces GST-P(+) liver foci also in non-initiated animals. Although limited, current data indicate that the genotoxic and carcinogenic potential of NOD might be even stronger than that of MCs.

CYN is an alkaloid, whose main target organ is the liver, but other organs (kidneys, lungs, thymus, spleen, adrenal glands, intestinal tract, the immune system and heart) can be affected as well. The principal mode of its action is the inhibition of protein and glutathione synthesis. Several studies imply that CYN is pro-genotoxic and needs CYP450-mediated metabolic activation to become genotoxic, suggesting that metabolites are responsible for its genotoxicity. Genotoxic effects of CYN include DNA adduct and strand break formation as well as aneugenic activity; there are no data available on its potential mutagenicity. CYN mediated carcinogenicity has not been studied extensively. Only one preliminary *in vivo* and one *in vitro* study are reported, showing CYN's tumor-initiating activity and cell transforming potential, respectively. Although there are not sufficient data available to assess its carcinogenic potential, the US EPA has classified CYN on the list of compounds with high priority for hazard characterization. Based on the evidence published in the literature and our own experimental data, CYN has much stronger genotoxic potential than MCs and NOD and should be considered as more dangerous to human and animal health.

Cyanobacterial neurotoxins ATX and homoanatoxin-a are potent post-synaptic depolarizing neuromuscular blocking agents. The acute neurotoxic effects of these neurotoxins are well explored; however, no data on their possible genotoxic and carcinogenic properties exist. Similarly, no experimental data for subchronic exposure, genotoxic and carcinogenic effects of freshwater STXs, a group of carbamate alkaloids, are available. Although it is generally hypothesized that these toxins do not represent significant concern regarding long-term effects, this hypothesis should be supported by adequate experimental data.

In addition to known toxins, cyanobacteria produce numerous bioactive compounds with currently unknown biological activities that may evoke toxic effects stronger than those of chemically characterized cyanotoxins. Furthermore, not much is known about the adverse effects of the interactions between cyanotoxins and other bioactive compounds and stressors present in cyanobacterial blooms. The limited data indicate possible synergisms.

6. Conclusion

We conclude that exposure to cyanobacterial toxins pose a serious threat for human and animal health. However, until now, a provisional guideline value in drinking water has been established only for the most extensively studied cyanotoxin, microcystin-LR, which was recently classified as a possible human carcinogen (Group 2B) by IARC. Unfortunately, other cyanobacterial toxins, nodularins, and especially cylindrospermopsins, as well as cyanobacterial extracts are currently not classifiable as carcinogens due to a lack of experimental data. However, there is ample indication they might possess even higher genotoxic and carcinogenic potential than MCLR. Therefore, in particular for NOD and CYN, new data are urgently needed on their mechanisms of carcinogenicity and carcinogenic potency to fully assess their genotoxic and especially carcinogenic potential. In addition, in the environment cyanobacterial toxins occur in complex mixtures as well as together with other anthropogenic contaminants, and numerous studies have found that the toxic/genotoxic potential of the extracts from cyanobacterial scums is higher than that of purified toxins. Thus research efforts should be focused on elucidation of the combined effects of the constituents. These studies should include thorough chemical characterization of the constituents of toxic cyanobacterial scums, which would also enable the identification and characterization of possible novel toxins.

Conflict of interest

The authors report no conflict of interest.

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2 ZNANSTVENA DELA

2.1 OBJAVLJENA ZNANSTVENA DELA

2.1.1 Genotoksični učinki cianobakterijskega hepatotoksina cilindrospermopsina pri celični liniji HepG2

Genotoxic effects of the cyanobacterial hepatotoxin cylindrospermopsin in the HepG2 cell line

Alja Štraser, Metka Filipič, Bojana Žegura

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Prisotnost cianobakterijskega alkaloida cilindrospermopsina (CYN) se vse pogosteje opaža v pitni vodi po vsem svetu. CYN je močan zaviralec sinteze proteinov in povzroča zastrupitve ljudi ter pogine živali. Objavljenih študij, ki preučujejo genotoksično delovanje CYN, je malo. Podatki večinoma kažejo, da je CYN pro-genotoksičen. V naši študiji smo preučevali genotoksičnost CYN pri celicah humanega hepatoma, HepG2, z analizo nastanka prelomov DNK verig, s testom komet, in analizo tvorbe mikrojeder (MNi), jedrnih brstov (NBUD) in nukleoplazmatskih mostičkov (NPB), s testom mikrojeder (CBMN). Poleg tega smo s kvantitativnim PCR v realnem času analizirali spremembe v izražanju genov, ki so vključeni v odziv na poškodbe DNK (*P53*, *CDKN1A*, *GADD45α* in *MDM2*), in genov, ki bi lahko sodelovali pri metabolični aktivaciji CYN (geni iz družine CYP450: *CYP1A1* in *CYP1A2*). Necitotoksične koncentracije CYN so povzročile poškodbe DNK po 12 in 24 h izpostavljenosti in povišale pogostost pojavljanja MNi, NBUDs in NPBs po 24 h izpostavljenosti. CYN je povišal izražanje genov *CYP1A1* in *CYP1A2*. Čeprav sprememb v izražanju tumor supresorskega gena *P53* nismo zaznali, je CYN povišal izražanje s P53 reguliranih genov *CDKN1A*, *GADD45α* in *MDM2*. Naši rezultati podajajo nove dokaze o genotoksičnem delovanju CYN in kažejo, da je ta toksin potrebno upoštevati pri oceni tveganja za zdravje ljudi.

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GENOTOXICITY AND CARCINOGENICITY

Genotoxic effects of the cyanobacterial hepatotoxin cylindrospermopsin in the HepG2 cell line

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Abstract The cyanobacterial alkaloid cylindrospermopsin (CYN) is being increasingly identified in drinking water supplies worldwide. It is a potent protein synthesis inhibitor and causes human intoxications and animal mortality. The few genotoxicity studies available indicate that CYN is genotoxic, generally implying that it is pro-genotoxic. We evaluated CYN genotoxicity in the human hepatoma cell line, HepG2, analyzing the induction of DNA strand breaks, with the alkaline comet assay, and micronuclei (MNi), nuclear bud (NBUD), and nucleoplasmic bridge (NPB) formation, with the cytokinesis block micronucleus (CBMN) assay. In addition, changes in the expression of genes involved in the response to DNA damage (*P53*, *CDKN1A*, *GADD45α*, and *MDM2*) and genes presumably involved in CYN metabolism (genes from the Cytochrome P450 family: *CYP1A1* and *CYP1A2*) were determined, using quantitative real-time PCR. Non-cytotoxic concentrations of CYN induced increased DNA damage after 12 and 24 h of exposure and increased the frequency of MNi, NBUDs, and NPBs after 24 h exposure. Moreover, CYN up-regulated the expression of the *CYP1A1* and *CYP1A2* genes. Although no changes in the expression of the *P53* tumor-suppressor gene were found, CYN up-regulated the expression of the P53 downstream-regulated genes *CDKN1A*, *GADD45α*, and *MDM2*. Our results provide new evidence that CYN is genotoxic and strongly suggest that it needs to be considered in the human health risk assessment.

Keywords Cylindrospermopsin · DNA damage · Micronucleus · Nuclear bud · Nucleoplasmic bridge · Gene expression

Abbreviations

BaP	Benzo[a]pyrene
BNC	Binucleated cells
CYN	Cylindrospermopsin
CYP450	Cytochrome p-450
CBMN	Cytokinesis block micronucleus assay
MNed	Micronucleated
MNi	Micronuclei
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NDI	Nuclear division index
NBUD	Nuclear bud
NPB	Nucleoplasmic bridge

Introduction

The cyanobacterial hepatotoxin cylindrospermopsin (CYN) has gained interest in recent years, since it is being increasingly recognized as a potential threat to drinking water safety. It was first identified as the probable cause of a severe case of human poisoning in Australia in 1979 (Byth 1980) and was isolated from the cyanobacterium *Cylindrospermopsis raciborskii* in 1992 (Ohtani et al. 1992). Nowadays, it is known to be produced by several other freshwater cyanobacterial species as well (Zegura et al. 2011). It is increasingly being found worldwide and was

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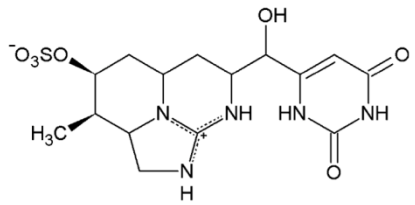

Fig. 1 Structure of cylindrospermopsin

reported to be implicated in several cases of human intoxications and animal mortality (Carmichael et al. 2001; Griffiths and Saker 2003; Hawkins et al. 1985). The main target organ of CYN activity is the liver, but other organs such as the kidney, lung, thymus, spleen, adrenal glands, intestinal tract, the immune system, and the heart can also be affected, after oral exposure (Falconer et al. 1999; Hawkins et al. 1985; Humpage et al. 2000). CYN is a potent protein synthesis inhibitor (Froscio et al. 2001, 2003; Runnegar et al. 2002), but there appear to be two different toxic responses (Falconer 2008). Its rapid toxicity seems to be mediated by cytochrome p-450 (CYP450)-generated metabolites, and the longer-term toxicity is most probably the consequence of the metabolite independent protein synthesis inhibition by CYN (Froscio et al. 2003; Humpage et al. 2005; Norris et al. 2002; Runnegar et al. 1995).

CYN is a stable 415 Da tricyclic alkaloid containing a guanido group linked at C7 to hydroxymethyl uracil through a hydroxyl bridge (Fig. 1) (Ohtani et al. 1992). The molecule contains several potential reactive sites that may form protein and DNA adducts, which has been also confirmed in vivo (Shaw et al. 2000). The presence of the uracil group in particular, indicates CYN could be interacting with adenine groups in RNA and DNA, interfering with DNA synthesis and could therefore induce mutations and act as a carcinogen (Falconer and Humpage 2001). Preliminary evidence has shown CYN could have tumor-initiating activity in a range of mice tissues (Falconer and Humpage 2001), and its cell transforming potential was shown in SHE cells (Maire et al. 2010).

In vitro (Bazin et al. 2010; Humpage et al. 2000, 2005) and in vivo (Shaw et al. 2000; Shen et al. 2002) genotoxicity studies have shown CYN to be genotoxic in a range of test systems (Zegura et al. 2011), generally implying that it is pro-genotoxic and needs to be activated by CYP450 enzymes (Bazin et al. 2010; Humpage et al. 2005). Although there is evidence for the genotoxic activity and carcinogenic potential of CYN, the mechanisms involved are not well understood. CYN has not been evaluated by the World Health Organization (WHO), but the U.S. Environmental Protection Agency (EPA) has classified CYN on the list of compounds with highest priority for hazard characterization (EPA 2001). Therefore, new data are urgently needed on its mechanisms of action to fully assess its genotoxic and especially carcinogenic potential.

The aim of this study was to investigate the potential genotoxic activity of CYN in the metabolically active human hepatoma cell line, HepG2. The HepG2 cells were used because they have retained inducibility and activities of several phase I and phase II xenobiotic metabolising enzymes and it has been shown that several classes of indirect acting genotoxic agents can be detected with this cell line (Knasmuller et al. 2004). In addition, HepG2 cells express wild-type tumor suppressor P53 (Bressac et al. 1990), making them an appropriate model for detecting P53 regulated response to DNA damage at the level of gene transcription. We analyzed DNA strand break formation, micronuclei (MN), nuclear bud (NBUD), and nucleoplasmic bridge (NPB) formation, and CYN-mediated modulation of the expression of genes involved in DNA damage response (*P53*, *CDKN1A*, *GADD45α*, and *MDM2*) and genes from the cytochrome P450 (CYP450) family presumably involved in CYN metabolism (*CYP1A1* and *CYP1A2*).

Materials and methods

Chemicals

Cylindrospermopsin (CYN) was from Enzo Life Sciences GmbH, Lausen, Switzerland. A 0.5 mg/ml stock solution of CYN was prepared in 50% methanol. William's medium E, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), benzo[a]pyrene (BaP), glycogen, cytochalasin B, and Ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma, St. Louis, USA. Penicillin/streptomycin, fetal bovine serum (FBS), L-glutamine, and phosphate buffered saline (PBS) were from PAA Laboratories, Dartmouth, USA. Triton X-100 was from Fisher Sciences, New Jersey, USA. Ethidium-bromide solution was from Promega, Madison, USA, and trypsin from BD-Difco, Le Pont-De-Claix Cedex, France. TRIzol reagent, normal melting point agarose (NMP), and low melting point agarose (LMP) were from Invitrogen, Paisley, Scotland, UK. cDNA High Capacity Archive Kit, TaqMan Universal PCR Master Mix and the Taqman Gene Expression Assays were from Applied Biosystems, New Jersey, USA. All other chemical reagents were of the purest grade available and all solutions were made using Mill-Q water.

Cell culture

HepG2 cells were a gift from Dr. Firouz Darroudi (Leiden University Medical Centre, Department of Toxicogenetics, Leiden, The Netherlands). The cells were grown at 37°C

and 5% CO_2 in William's medium E (Sigma, St. Louis, USA) containing 15% fetal bovine serum, 2 mM L-glutamine and 100 U/ml penicillin/streptomycin.

Cell viability: MTT assay

The cytotoxicity of CYN was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) according to Mosmann (Mosmann 1983) with minor modifications (Zegura et al. 2008). The HepG2 cells were seeded onto 96-well microplates (Nunc, Naperville IL, USA) at a density of 8,000 cells/well and incubated for 20 h at 37°C in 5% CO_2 to attach. Growth medium was replaced with fresh medium containing 0, 0.005, 0.01, 0.05, 0.1, 0.5, 1, and 5 µg/ml of CYN. Statistical significance between treated groups and the control was determined by two tailed Student's *t* test, and $P < 0.01$ was considered significant. Independent experiments were performed in 5 replicates and repeated three times.

Alkaline comet assay

HepG2 cells were seeded at a density of 60 000 cells/well into 12-well microtiter plates (Corning Costar Corporation, Corning, NY, USA). After incubation at 37°C in 5% CO_2 for 20 h, the growth medium was replaced with fresh medium containing 0, 0.005, 0.01, 0.05, 0.1, and 0.5 µg/ml CYN and incubated for 4, 12, and 24 h. In each experiment, a positive control (30 µM BaP) and a vehicle control (cell growth medium containing 0.05% methanol) were included. At the end of the exposure, the cells were harvested and the DNA damage was determined as described by Singh et al. (Singh et al. 1988) with minor modifications (Zegura et al. 2004). Images of 50 randomly selected nuclei per experimental point were analyzed with image analysis software Comet Assay IV (Perceptive Instruments, UK). Three independent experiments were performed for each of the treatment conditions. One-way analysis of variance (1-way ANOVA) was used to analyze the differences in the percent of tail DNA between treatments within each experiment. Dunnett's Multiple Comparison test was used for comparing the sample groups to the control group; $P < 0.05$ was considered as statistically significant.

Cytokinesis block micronucleus assay (CBMN)

HepG2 cells were seeded on T-25 flasks (Corning Costar Corporation, Corning, NY, USA) (700 000 cells/plate) and incubated for 20 h at 37°C and 5% CO_2. They were then exposed to 0.005, 0.05, and 0.5 µg/ml CYN for 24 h. BaP (10 µM) was used as the positive control and 0.05% methanol as the vehicle control. After the treatment, the cells were washed twice with 1× PBS and cytochalasin B (final concentration 2 µg/ml) was added to the cells. After subsequent 26 h of incubation at 37°C, the medium containing cytochalasin B was removed and the cells were washed with 1× PBS. The cells were then trypsinized and incubated in cold hypotonic solution (75 mM KCl) for 5 min and fixed with methanol/acetic acid (3/1) (v/v) and formaldehyde. The fixation was repeated 3 times. The fixed cells were put on microscope slides, air dried, stained with acridine orange (20 µg/ml), and examined under the fluorescence microscope (Eclipse 800, Nikon, Japan). Micronuclei (MNi), nuclear buds (NBUDs), and nuclear bridges (NPBs) were counted in 1,000 binucleated cells (BNC) per experimental point at 400× magnification (Fig. 2) and were scored according to criteria published by Fenech (Fenech 2000). The nuclear division index (NDI) was estimated by scoring 500 cells with one to four nuclei. The NDI was calculated using the formula [M1 + 2M2 + 3(M3 + M4)]/500, where M1–M4 represent the number of cells with one to four nuclei, respectively. Experiments were repeated three times, independently. Student's *t* test was used to compare the number of MNi, NBUDs, or NPBs between vehicle control and CYN exposed cells; $P < 0.05$ was considered as statistically significant.

mRNA expression analysis

Cells were seeded on T-25 flasks (Corning Costar Corporation, Corning, NY, USA) at a density of 10^6 cells/flask and incubated for 20 h at 37°C and 5% CO_2. They were washed with 1× PBS, exposed to 0.005, 0.05, and 0.5 µg/ml CYN and incubated for 4, 12, and 24 h. In each experiment, a positive control (30 µM BaP) and a vehicle control (0.05% methanol) were included. After the incubation, cells were washed twice with 1× PBS and total RNA was isolated using TRIzol reagent according to the manufacturer's protocol with minor modifications. Glycogen (20 µg/ml) was added to the cell lysate. The RNA was incubated with isopropyl alcohol overnight at −20°C to precipitate. All solutions needed for RNA isolation were prepared in RNase-free water.

The RNA was transcribed to cDNA using 1 µg of total RNA and cDNA High Capacity Archive Kit, according to the manufacturer's protocol. Gene expression was quantified using real-time quantitative PCR (ABI 7900 HT Sequence Detection System, Applied Biosystems, USA). TaqMan Universal PCR Master Mix and the following Taqman Gene Expression Assays were used: *P53* (tumor protein p53), Hs00153349_m1; *CDKN1A* ('cyclin-dependent kinase inhibitor 1A') Hs00355782_m1; *GADD45α* ('growth arrest and DNA damage-inducible gene, alpha'), Hs00169255_m1; *MDM2* (Mdm2, 'transformed 3T3 cell double minute 2', p53 binding protein gene), Hs00234753_m1; *CYP1A1* (cytochrome P450, family 1, subfamily A, polypeptide 1) Hs00153120_m1; and *CYP1A2*

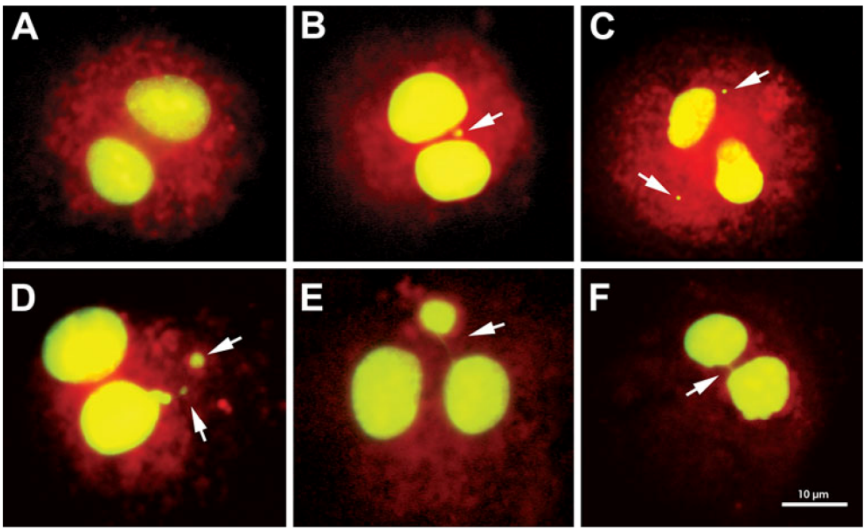

Fig. 2 Flourescent micrographs of HepG2 cells exposed to CYN (0.5 µg/ml for 24 h) **a** binucleated cell, **b** binucleated cell with MN, **c** binucleated cell with two MNi, **d** binucleated cells with NBUD and MN, **e** binucleated cells with NBUD, and **f** binucleated cell with NBP

(cytochrome P450, family 1, subfamily A, polypeptide 2) Hs01070374_m1. Amplification of the *GAPDH* gene was performed as an internal control. The conditions for the PCR were 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. The data obtained from Taqman Gene Expression Assays were analyzed using the ΔΔCt algorithm. Statistical significance between treated groups and controls was determined by two tailed Student's *t* test, and $P < 0.01$ was considered significant. Two independent experiments were performed, each on duplicate samples.

Results

Cytotoxicity of CYN in HepG2 cells

CYN significantly affected cell viability at concentrations 1 µg/ml (2 µM) and above, reducing cell viability for more than 30% (Fig. 3); therefore, concentrations of up to 0.5 µg/ml were used for further experiments.

Induction of DNA strand breaks

DNA damage in HepG2 cells induced by CYN was determined with the comet assay (Fig. 4). No significant increase in the amount of DNA strand breaks was observed after 4 h of exposure to CYN (Fig. 5a). Statistically significant increase in DNA damage was detected after 12 h and 24 h exposure to 0.5 µg/ml (Fig. 5b) and 0.01, 0.05, 0.1, and 0.5 µg/ml CYN (Fig. 5c), respectively.

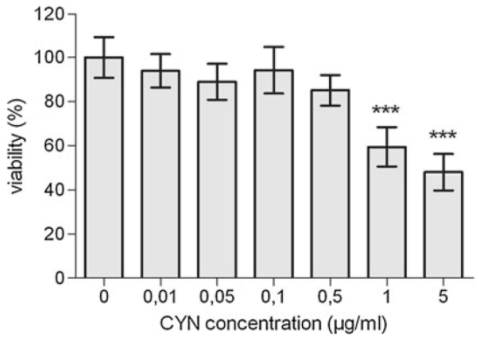

Fig. 3 The effect of CYN on the viability of HepG2 cells. The viability was determined with the MTT assay after the exposure to different concentrations of CYN (0.005–5 µg/ml) for 24 h. *** denotes significant difference between the vehicle control (0) and CYN-treated cells (Student's *t* test, $P < 0.001$)

Induction of micronuclei, nucleoplasmic bridges, and nuclear buds

The genotoxic activity of CYN in HepG2 cells was further evaluated using the CBMN assay. Following 24 h exposure, CYN induced dose–dependent increase in the frequencies of MNi, NBUDs, and NBPs in BNCs (Fig. 6). Statistically significant increase in the frequencies of MNi (Fig. 6a) and MNed cells (Fig. 6b) were detected at 0.05 and 0.5 µg/ml CYN ($P < 0.05$) and were about 1.9-fold and more than threefold higher than in the control cells, respectively. The frequency of NBUDs (Fig. 6c) was statistically significant at 0.05 and 0.5 µg/ml CYN ($P < 0.05$) and was

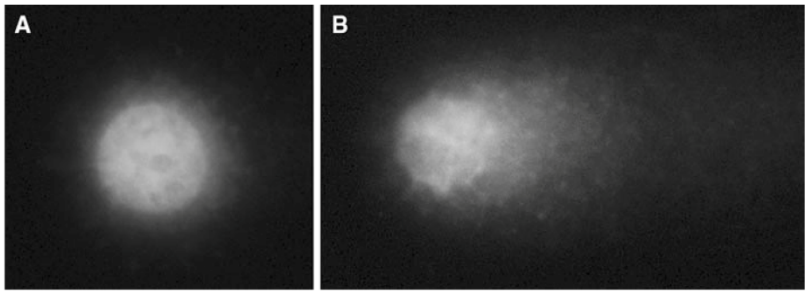

Fig. 4 Comet assay images; **a** undamaged nucleus (8.39% tail DNA) from the unexposed population **b** damaged nucleus (54.69% tail DNA) from the cell population exposed to CYN (0.5 µg/ml for 24 h)

1.7- and 2.6-fold higher than in the control cells, respectively. The increase in NPB frequency (Fig. 6d) was detected at 0.05 and 0.5 but was statistically significant ($P < 0.05$) only at 0.05 µg/ml CYN. Statistically significant decrease ($P < 0.05$) in NDI was determined in cells exposed to 0.5 µg/ml of CYN (Fig. 7). BaP (10 µM) induced statistically significant increased frequencies of MNed cells (3.5-fold), MNi (3.9-fold), NBUDs (1.9-fold), but not NPBs.

Effect of CYN on changes in gene expression

The expression of selected DNA damage responsive genes, and cytochrome P450 genes *CYP1A1* and *1A2* were analyzed after 4, 12, and 24 h exposure of HepG2 cells to 0.005, 0.05, and 0.5 µg/ml of CYN by quantitative real-time PCR (Table 1).

Exposure to CYN did not induce any changes in the mRNA expression of *P53*, (Table 1) while its down-stream-regulated genes *CDKN1A*, *GADD45α*, and *MDM2* (Table 1) were significantly up-regulated at the highest concentration of CYN (0.5 µg/ml). Up-regulation of *CDKN1A* was observed after 24 h exposure to CYN and was 2.3-fold higher than in the control cells. The expression of *GADD45α* was up-regulated after 12 h by 1.3-fold at the highest concentration of CYN, and after 24 h exposure, its expression was almost 4.8-fold higher than in the control cells. Slight but significant up-regulation of *MDM2* was observed after 12 (1.3-fold) and 24 h (1.6-fold) exposure to CYN. In cells exposed to 30 µM BaP, which was used as the positive control, the mRNA expression of *P53* was not changed; the expression of *CDKN1A* was up-regulated by 4.5- and 11.0-fold after 12 and 24 h exposure, respectively, and *GADD45α* by 2.2- and 6.2-fold, respectively, while *MDM2* was down-regulated at all three time points.

The expression of *CYP1A1* and *CYP1A2* was up-regulated in cells exposed to the highest concentration of CYN (0.5 µg/ml). Compared with the vehicle treated, control, cells the expression of *CYP1A1* was up-regulated by 3.6- and 13.9-fold after 12 and 24 h exposure, respectively, whereas 3.6-fold increase in *CYP1A2* expression was observed after 24 h exposure. In BaP exposed cells, the expression of *CYP1A1* was increased by 2.4-, 26-, and 124-fold after 4, 12, and 24 h exposure, respectively. The expression of *CYP1A2* was 1.3-, 2-, and tenfold higher after 4, 12, and 24 h exposure, respectively, compared with control cells.

Discussion

In this study, we showed that CYN is genotoxic in the human hepatoma HepG2 cells. At non-cytotoxic concentrations, CYN induced DNA breakage, MNi, NBUD, and NPB formation that were associated with the up-regulation of DNA damage responsive genes *CDKN1A*, *GADD45a*, and *MDM2*. We also showed that exposure of HepG2 cells to CYN induced the up-regulation of cytochrome P450 genes *CYP1A1* and *CYP1A2* providing further evidence that these enzymes are involved in metabolic activation of CYN. The observed induction of genotoxicity is in accordance with several previous studies showing CYN induced DNA damage in mouse primary hepatocites in vitro (Humpage et al. 2005) and in vivo in mice liver cells (Shen et al. 2002), while CYN induced MNi formation has been previously reported in three human cell lines; the lymphoblastoid cell line, WIL2-NS (Humpage et al. 2000), liver-derived HepaRG cells, and colon-derived Caco-2 cells (Bazin et al. 2010).

It is generally accepted that toxicity and genotoxicity of CYN are CYP-450–metabolism dependent as has been indicated by the protective effects of CYP450 inhibitors against its toxicity (Froscio et al. 2003; Norris et al. 2002; Runnegar et al. 1995) and genotoxicity (Bazin et al. 2010; Humpage et al. 2005) as well as the lack of genotoxic effects in Chinese hamster ovary cells, which have low activity of metabolizing enzymes (Fessard and Bernard 2003; Lankoff et al. 2007). In HepG2 cells, we observed the induction of DNA damage and MNi formation within the dose range of 0.01–0.5 µg/ml, at which increase in DNA strand breaks was observed also in mouse primary hepatocytes (Humpage et al. 2005), indicating that the CYN-metabolizing capacity of HepG2 cells may be comparable to that of primary hepatocytes. Within the same dose range

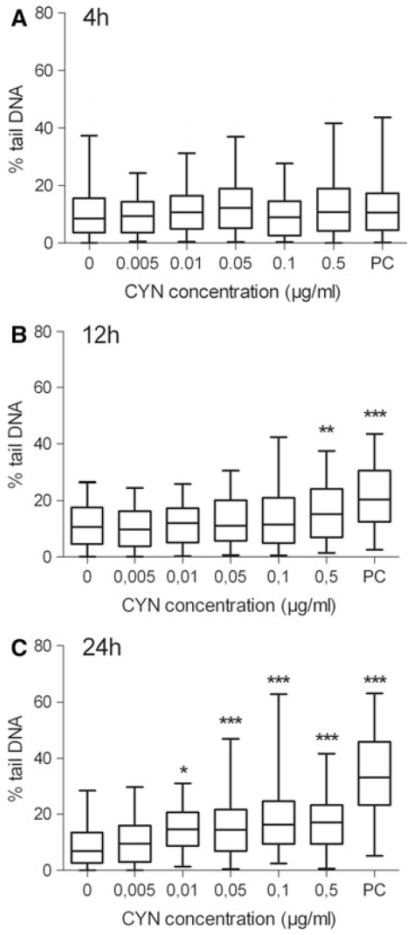

Fig. 5 CYN induced DNA damage in HepG2 cells. Cells were exposed to CYN (0.005–0.5 µg/ml) for 4 h (**a**), 12 h (**b**) and 24 h (**c**). Benzo[a]pyrene (30 µM) was used as the positive control (PC). DNA damage was assessed with the comet assay and is expressed as percent of tail DNA. Fifty cells were analyzed per experimental point in each of the three independent experiments. Data are presented as quantile box plots. The edges of the box represent the 25th and 75th percentiles, the median is a solid line through the box, and the error bars represent 95% confidence intervals. Significant difference (1-way ANOVA; Dunnett's Multiple Comparison test) between CYN-treated cells and the vehicle control (0) is indicated by $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$

of CYN (0.04–0.2 µg/ml), MNi induction has been detected also in differentiated HepaRG cells that express metabolic enzymes at levels comparable with those of primary human hepatocytes, while in undifferentiated HepaRG cells that express metabolic enzymes at a lower level, no MNi were induced (Bazin et al. 2010). Induction of MNi was observed also in differentiated and un-differentiated Caco-2 cells that also express CYP-450 enzymes; however, these cells were less sensitive, and MNi induction was observed at higher doses of CYN (0.5–2 µg/ml) (Bazin et al. 2010). The authors explained the higher sensitivity of differentiated compared with undifferentiated HepaRG cells and Caco-2 cells by higher metabolic activity of differentiated HepaRG cells. CYN induced MNi formation was observed also in WIL2-NS cells, for which their metabolic activity is not known, however, at much higher doses (1–10 µg/ml) (Humpage et al. 2000). In this study, also centromere staining by fluorescent in situ hybridization (FISH) has been applied and the results showed that CYN induced both centromere positive and centromere negative MNi indicating that it has clastogenic and aneugenic activity (Humpage et al. 2000).

Despite the evidence that CYP-450 family enzymes are involved in the metabolic activation of CYN it is not clear which isoforms are involved. In mouse primary hepatocytes complete prevention of CYN induced DNA strand breaks has been observed in the presence of the broad-spectrum monooxigenase inhibitor SKF525A or in the presence of omeprazole (Humpage et al. 2005), whereas ketoconazole, a potent competitive CYP3A4 inhibitor, attenuated CYN induced MNi formation in Caco-2, but not in HepaRG cells (Bazin et al. 2010). However, ketoconazole is also a potent inhibitor of CYP1A1 and a moderate inhibitor of CYP1A2, CYP2C, and CYP2D6 (Pelkonen et al. 2008), thus exclusive role of CYP3A4 in the metabolic activation of CYN could not be confirmed (Bazin et al. 2010). In our study, we observed time-dependent up-regulation of the expression of *CYP1A1* and *1A2* that correlated with the time-dependent increase in DNA damage, and with the time-dependent up-regulation of the expression of the DNA damage responsive genes. The CYP1A1 and 1A2 isoforms are highly inducible enzymes, widely known as activators of numerous carcinogenic compounds, and here, we provide first evidence that exposure to CYN induces transcription of these enzymes, supporting previous assumptions that they are involved in CYN metabolic activation to genotoxic intermediate(s).

The CBMN assay was originally developed as a method for measuring micronuclei (MNi) formation and it has only recently been proposed that measuring nucleoplasmic bridges (NPB) and nuclear buds (NBUD) formation may give additional information on the extent of genetic instability (Fenech 2006). It has been proposed that NBUDs are formed during S-phase, representing extrusion of either amplified DNA (Shimizu et al. 1998) or chromatin, whose replication has failed (Yankiwski et al. 2000), thus they are considered as an indicator of gene amplification and/or DNA repair complexes. It has been shown that NBUDs and MN are formed simultaneously after exposure to genotoxins with positive correlation factors between 0.73 and 0.84 (Serrano-García and Montero-Montoya 2001). NPBs are thought to be markers of DNA mis-repair, chromosome rearrangements or telomere end-fusions and are suggested to originate from dicentric chromosomes in which the

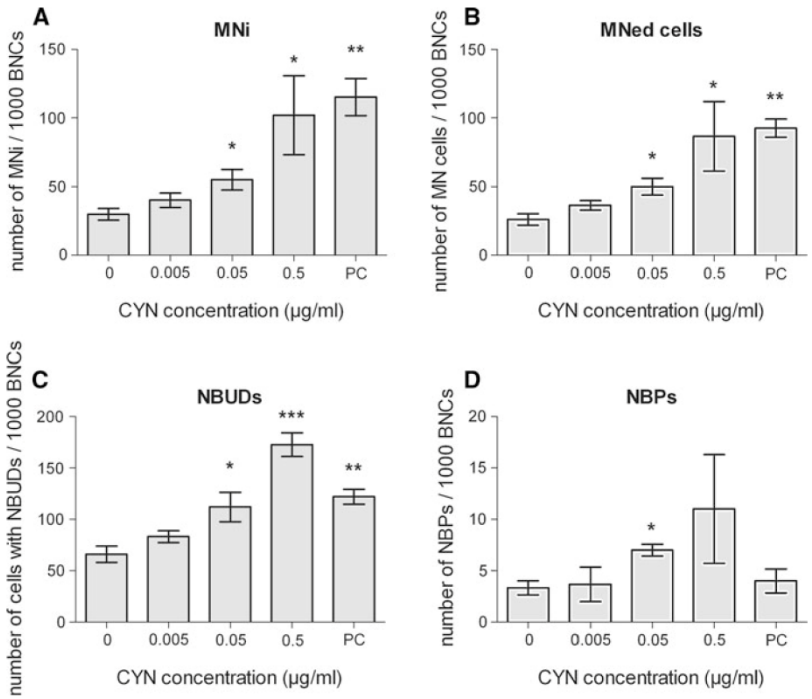

Fig. 6 CYN influence on the frequency of MNi (**a**), MNed cells (**b**), NPBs (**c**), and NBUDs (**d**) in HepG2 cells. Cells were exposed to CYN (0.005–0.5 µg/ml) for 24 h. Benzo[a]pyrene (10 µM) was used as the positive control (PC). Thousand binucleated cells were counted at each experimental point. Significant difference (Student's *t* test) between CYN-treated cells and the vehicle control (0) is indicated by $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$

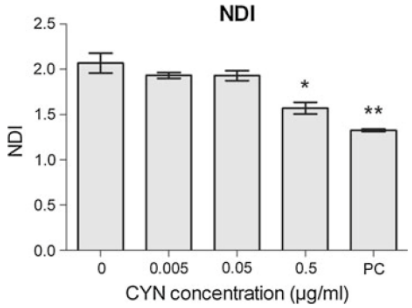

Fig. 7 CYN influence on the nuclear division index (NDI) in HepG2 cells. Cells were exposed to CYN (0.005–0.5 µg/ml) for 24 h. Benzo[a]pyrene (10 µM) was used as the positive control (PC). The NDI was estimated by scoring 500 cells with one to four nuclei. Significant difference (Student's *t* test) between CYN-treated cells and the vehicle control (0) is indicated by $*P < 0.05$ and $**P < 0.01$

centromeres have been pulled to the opposite cell poles at anaphase (Fenech 2000). It has been shown that the incidence of NPBs might be a sensitive indicator of chromosomal structural rearrangements (Thomas et al. 2003). As shown in Fig. 6 in HepG2 cells CYN induced NBUD and NBP formation, which correlates with the increase in MNi formation, indicating that CYN induces complex genomic alterations including gene amplification and structural chromosomal rearrangements. This is new information as to our knowledge, the effect of CYN on the induction of NBUD and NPB formation has not been studied before.

As an addition to conventional genotoxicity assays, toxicogenomic analysis are becoming widely used as a tool for hazard identification and risk assessment of genotoxic and carcinogenic properties of different substances (Ellinger-Ziegelbauer et al. 2009). Changes in the expression of *p53* and the p53-regulated DNA damage response genes, like *cdkn1a*, *mdm2*, and *gadd45α*, can be considered as markers of genotoxic and carcinogenic stress (Vogelstein et al. 2000). The tumor-suppressor gene, *p53*, plays the central role in the cellular response to agents or conditions that damage DNA by activating the transcription of several essential genes controlling cell cycle arrest/DNA repair, senescence, differentiation and apoptosis (Vogelstein et al. 2000). The concentration of p53 in the cell is regulated mainly through a negative feedback loop. Elevated levels of p53 up-regulate the expression of *mdm2*, which encodes mdm2, an ubiquitine-ligase, that mediates ubiquitination of p53, targeting it for proteosomal degradation (Michael and Oren 2002; Vogelstein et al. 2000). The main target of P53 upon DNA damage is *cdkn1a* that encodes $p21^{WAF1/CIP1}$, an inhibitor of cyclin-dependent kinases (CDKs) that inhibits the cell cycle at the G1-S and the G2-M transitions (Michael and Oren 2002; Vogelstein et al. 2000). The *gadd45α* gene is induced by genotoxic stress through p53-dependent and p53-independent pathways (Smith et al. 1994). Its product plays an important role in the control of cell cycle G2-M checkpoint (Jin et al. 2000; Wang et al. 1999), induction of cell death (Harkin et al. 1999;

Table 1 Changes in gene expression of *P53*, *MDM2*, *GADD45α*, *CDKN1A*, *CYP1A1*, and *CYP1A2* after exposure of HepG2 cells to CYN (0.005–0.5 μg/ml) for 4, 12, and 24 h

Gene	CYN concentration (μg/ml)	4 h	12 h	24 h
P53	0	1.002 ± 0.076	1.002 ± 0.071	1.000 ± 0.035
	0.005	1.040 ± 0.073	0.946 ± 0.014	1.007 ± 0.057
	0.05	0.957 ± 0.065	0.955 ± 0.065	0.898 ± 0.058
	0.5	0.970 ± 0.131	0.882 ± 0.053	0.967 ± 0.043
	PC	0.964 ± 0.056	1.475 ± 0.602	1.113 ± 0.111
CDKN1A	0	1.001 ± 0.046	1.000 ± 0.034	1.002 ± 0.066
	0.005	0.929 ± 0.026	1.039 ± 0.129	1.110 ± 0.206
	0.05	0.978 ± 0.088	0.931 ± 0.088	0.950 ± 0.066
	0.5	0.999 ± 0.033	1.138 ± 0.126	**2.291 ± 0.222*****
	PC	1,294 ± 0,268	4,494 ± 1,566*	10,985 ± 0,582***
GADD45α	0	1.002 ± 0.065	1.001 ± 0.049	1.001 ± 0.042
	0.005	0.870 ± 0.075	0.935 ± 0.308	1.266 ± 0.112
	0.05	1.036 ± 0.033	0.826 ± 0.090	0.954 ± 0.066
	0.5	1.054 ± 0.036	**1.287 ± 0.090****	**4.783 ± 0.611*****
	PC	0,464 ± 0,101***	2,240 ± 0,908*	6,164 ± 1,111***
MDM2	0	1.007 ± 0.133	1.001 ± 0.051	1.002 ± 0.075
	0.005	0.791 ± 0.066	1.047 ± 0.189	1.112 ± 0.030
	0.05	0.775 ± 0.093	0.985 ± 0.094	0.990 ± 0.016
	0.5	0.922 ± 0.101	**1.288 ± 0.185***	**1.601 ± 0.251****
	PC	0,690 ± 0,039**	0,632 ± 0,075	0,701 ± 0,028***
CYP1A1	0	1.000 ± 0.039	1.001 ± 0.040	1.003 ± 0.090
	0.005	1.076 ± 0.016	0.909 ± 0.029	1.053 ± 0.039
	0.05	1.099 ± 0.075	1.041 ± 0.131	1.133 ± 0.285
	0.5	**1.140 ± 0.102***	**3.693 ± 0.297*****	**11.771 ± 7.161***
	PC	2.403 ± 0.677**	26.483 ± 16,006*	124,064 ± 58,829**
CYP1A2	0	1.001 ± 0.061	1.002 ± 0.068	1.007 ± 0.140
	0.005	1.016 ± 0.055	0.834 ± 0.101	1.026 ± 0.331
	0.05	0.898 ± 0.096	0.872 ± 0.048	1.340 ± 0.587
	0.5	1.006 ± 0.077	0.920 ± 0.066	**3.595 ± 1.646***
	PC	1,257 ± 0,053***	2,024 ± 0,117***	10,947 ± 1,581***

The asterisk denote a significant difference between CYN-treated groups and the vehicle control (0) (Student's *t* test; $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$). PC is the positive control group, where the cells were exposed to BaP 30 μM

The results are expressed as mRNA expression fold-change of the vehicle control (0) ± STD (ΔΔCt algorithm)

Takekawa and Saito 1998), and the DNA repair process (Hollander et al. 2001; Smith et al. 1994, 1996).

In the present study, we measured changes in the expression of P53 and its downstream regulated DNA damage response genes *CDKN1A, GADD45α*, and *MDM2* at the same time points as DNA damage. We could not detect any change in the expression of *P53,* at any tested concentration and time of exposure to CYN. This is not unusual, as it is known that DNA damage activates the p53 protein predominantly through its phosphorylation by DNA damage-responsive kinases and, to lesser extent, through up-regulation of gene expression (Zhou and Elledge 2000). Also Bain et al. (Bain et al. 2007) did not detect any changes in the expression of P53 at the transcriptional level, in a previous study, with HepG2 cells exposed to CYN, whereas P53 protein accumulation was detected after 48 h exposure. In our study, no changes in gene expression were observed after 4 h exposure to CYN, whereas at the highest tested concentration (0.5 μg/ml) after 12 h exposure elevated expression of *GADD45α* and *MDM2* was detected, and after 24 h of exposure also expression of *CDKN1A* was increased, which correlates with the time-dependent increase in DNA damage. The up-regulation of *CDKN1A* and *GADD45α* can be also associated with the cell cycle arrest upon DNA damage, which may explain the NDI reduction seen with the CBMN assay at the highest concentration of CYN. Our study corroborates the study by Bain et al. (Bain et al. 2007), who detected up-regulation of *CDKN1A*, *GADD45α*, *MDM2*, and *BAX* in HepG2 cells and in human dermal fibroblasts exposed to CYN (1–5 μg/ml) for 6 and 24 h, however, the concentrations used in their study were much higher than in ours. The observed changes in the expressions of DNA damage responsive genes, which we show to correlate with the time-dependent increase in

induction of DNA damage as well as with the increase in the expression of the metabolic enzyme genes *CYP1A1* and *CYP1A2*, give additional evidence that CYN is an indirect acting genotoxin.

Conclusions

In conclusion, exposure of HepG2 cells to CYN induced increase in DNA damage, MNi, NBUD, and NPB formation as well as up-regulation of DNA damage responsive genes *CDKN1A*, *GADD45α*, and *MDM2*, confirming its genotoxic activity. The elevation of the mRNA expression of the genes of the two metabolic enzymes *CYP1A1* and *CYP1A2* gives evidence that CYN is an indirect acting genotoxin. These results present valuable information on the potential hazard of CYN, especially because the effects were detected at concentrations that have been detected in environmental samples (Saker and Eaglesham 1999; Shaw et al. 2000). Nevertheless, the aim of this in vitro study was to elucidate the mechanisms of CYN genotoxicity and the concentrations of CYN to which HepG2 cells were exposed although at the levels found in the environment are not likely to be reached in the liver of exposed individuals. On the other hand, HepG2 cells posses lower metabolic enzyme activity than primary human hepatocytes (Westerink and Schoonen 2007). We must be aware that the prediction of the hazardous effects of CYN for human health from our results could be underestimated as HepG2 cells are probably less sensitive to CYN than human hepatocytes. Therefore, further in vitro as well as in vivo studies are needed to characterize the hazard of CYN.

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Conflict of interest The authors report no conflict of interest.

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2.1.2 Cilindrospermopsin povzroča poškodbe DNK in spremembe v izražanju genov, vpletenih v odziv na poškodbe DNK, apoptozo in oksidativni stres

Cylindrospermopsin induced DNA damage and alteration in the expression of genes involved in the response to DNA damage, apoptosis and oxidative stress

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Cilindrospermopsin (CYN), močan cianobakterijski citototoksin, povezan z zastrupitvijo ljudi in pogini živine, ki ga sintetizirajo določene sladkovodne cianobakterije, redno odkrivajo v vodnih zajetjih v mnogih delih sveta. V študijah, kjer so preučevali genotoksičnost CYN, so pokazali, da deluje genotoksično in da je pro-genotoksin. Na humanih limfocitih iz periferne krvi (HPBL) smo s testom komet pokazali, da CYN (0, 0,05, 0,1 in 0,5 mg /ml) povzroča nastanek DNK prelomov. Po izpostavitvi HPBL CYN smo opazili statistično značilno, od koncentracije in časa odvisno, povišanje nastanka mikrojeder (MNI) in jedrnih brstov (NBUD), medtem ko se je število nukleoplazmatskih mostičkov (NPB) le rahlo povišalo. Analizirali smo tudi spreminjanje izražanja genov v HPBL po izpostavitvi CYN (0,5 mg/ml) z uporabo kvantitativnega PCR v realnem času. Izražanje genov, ki so verjetno vključeni v metabolično aktivacijo CYN (*CYP1A1* in *CYP1A2*), je bilo po izpostavitvi CYN povišano. CYN je povzročil tudi spremembe v izražanju gena *P53* in z njim reguliranih genov, vpletenih v odgovor na poškodbe DNK (*MDM2*, *GADD45a*) in apoptozo (*BCL-2* in *BAX*), kot tudi v odziv na oksidativni stres (*GPX1*, *SOD1*, *GSR*, *GCLC*). Sprememb v izražanju genov *CDKN1A* in *CAT* nismo zaznali. Ti rezultati kažejo, da je treba CYN obravnavati kot genotoksično snov in da so lahko tudi limfociti tarča njegovega genotoksičnega delovanja.

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Cylindrospermopsin induced DNA damage and alteration in the expression of genes involved in the response to DNA damage, apoptosis and oxidative stress

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ABSTRACT

Cylindrospermopsin (CYN), a potent cyanobacterial cytototoxin produced by certain freshwater cyanobacteria, is regularly found in water supplies in many parts of the world, and has been associated with the intoxication of humans and livestock. The few genotoxicity studies available indicate that CYN is genotoxic, generally implying that it is progenotoxic. In human peripheral blood lymphocytes (HPBLs) CYN (0, 0.05, 0.1 and 0.5 μg/ml) induced the formation of DNA single strand breaks, applying the comet assay. Time and dose dependent significant increase in the frequency of micronuclei and nuclear buds was observed after the exposure of HPBLs to CYN, while there was only slight increase in the number of nucleoplasmic bridges. For the first time the modulation of gene expression in HPBLs was studied after the exposure to CYN (0.5 μg/ml), using the quantitative real-time PCR. The genes presumably involved in CYN metabolism (*CYP1A1* and *CYP1A2*) were up-regulated after the exposure. CYN induced changes in the mRNA expression of *P53* and its downstream regulated DNA damage responsive genes *MDM2*, *GADD45α* and apoptosis genes, *BCL-2* and *BAX*, as well as oxidative stress responsive genes (*GPX1*, *SOD1*, *GSR*, *GCLC*), while no changes in the expression of genes *CDKN1A* and *CAT* were observed. These results provide strong evidence that CYN should be considered as genotoxic and that lymphocytes can also be a target of cylindrospermopsin induced genotoxicity.

1. Introduction

The cyanobacterial cytotoxin cylindrospermopsin (CYN) is synthesized by a number of freshwater cyanobacterial species including *Cylindrospermopsis raciborskii* (Li et al., 2001a; Ohtani et al., 1992), *Anabaena bergii* (Schembri et al., 2001), *Anabaena lapponica* (Spoof et al., 2006), *Umezakia natans* (Terao et al., 1994), *Aphanizomenon ovalisporum* (Banker et al., 1997; Shaw et al., 1998), *Aphanizomenon flos-aquae* (Preußel et al., 2006) and *Raphidiopsis curvata* (Li et al., 2001b).

It is a stable 415 Da tricyclic alkaloid containing a guanido group linked at C7 to hydroxymethyl uracil through a hydroxyl bridge (Ohtani et al., 1992). Because of the negatively charged sulfate group and the positively charged guanido group, the molecule is a zwitterion and very water soluble (Falconer and Humpage, 2006).

CYN can be found worldwide in drinking-water sources such as lakes and reservoirs and has been implicated in human intoxications and animal mortality (Carmichael et al., 2001; Griffiths and Saker, 2003; Hawkins et al., 1985). The first human poisoning connected to CYN is known as the

Abbreviations: BNC, binucleated cells; CYN, cylindrospermopsin; CYP450, cytochrome p-450; CBMN, cytokinesis-block micronucleus assay; MNed, micronucleated; MN, mucronucleus; MNi, micronuclei; NDI, nuclear division index; NBUD, nuclear bud; NPB, nucleoplasmic bridge.

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"Palm Island Mystery Disease" from 1979, when 148 cases of severe hepatoenteritis with renal tubular damage were recorded on an island off Queensland, Australia, coast (Byth, 1980; Hawkins et al., 1985). In combination with another cyanobacterial toxin, microcystin, it was implicated in the most tragic outbreak of acute liver failure and death of 76 patients from dialysis center in Caruaru, Brazil in 1996 (Carmichael et al., 2001).

The main target organ of CYN activity is the liver, but other organs such as the kidneys, lungs, thymus, spleen, adrenal glands, intestinal tract, the immune system and the heart can be affected as well (Falconer et al., 1999; Hawkins et al., 1985; Humpage et al., 2000; Terao et al., 1994). The principal mode of action of CYN is the inhibition of protein synthesis (Froscio et al., 2003, 2001; Runnegar et al., 2002) and glutathione synthesis (Humpage et al., 2005; Runnegar et al., 1995, 1994). However, the rapid toxicity of this compound seems to be mediated by cytochrome P-450-generated metabolites rather than the inhibition of protein synthesis, which presents long-term toxicity (Froscio et al., 2003; Humpage et al., 2005; Norris et al., 2002; Runnegar et al., 1995).

CYN has been found to be genotoxic in a number of *in vitro* studies (Bazin et al., 2010a; Humpage et al., 2005, 2000; Shen et al., 2002; Straser et al., in press) and *in vivo* studies (Bazin et al., 2010b; Shen et al., 2002). Although there is evidence for the genotoxic activity and carcinogenic potential of CYN, the mechanisms involved are not well understood. CYN is at present being included in the revision of the World Health Organization (WHO) Guidelines for Drinking-water Quality, chemical hazards in drinking-water and U.S. Environmental Protection Agency (EPA) has classified CYN on the list of compounds with the highest priority for hazard characterization (EPA, 2001). The information regarding the genotoxic activity of CYN is still scarce and unclear; therefore, it is very important to elucidate the mechanisms leading to its genotoxicity and potential carcinogenicity, especially as CYN is probably more hazardous to human and animal health than microcystins (Zegura et al., 2011).

The aim of this study was to investigate the ability of CYN to induce DNA damage by measuring DNA strand breaks and micronuclei formation in isolated primary human lymphocytes. In addition, the mechanisms of CYN induced genotoxicity were evaluated by examining the gene expression patterns of selected genes involved in metabolism (*CYP1A1* and *CYP1A2)*, DNA damage responsive genes (*P53*, *MDM2*, *GADD45α, CDKN1A)*, genes involved in oxidative stress (*GPX1*, *SOD1*, *GSR*, *GCLC*, *CAT*) and apoptosis (*BCL-2* and *BAX)*, using real-time quantitative PCR (QRT-PCR).

2. Materials and methods

2.1. Chemicals

Chromosome kit P was from Euroclone, Italy; fetal bovine serum, RPMI 1640, TRIzol reagent were from Invitrogen, Carlsbad, USA; penicillin/streptomycin, cytochalasin B, histopaque, ethidium bromide, low melting point (LMP) and normal melting point (NMP) agaroses were from Sigma, USA; phytohaemagglutinin was from Remel Europe Ltd, England; heparinised vacutainer tubes from Becton Dickinson, USA; Giemsa from Merk, Germany; cylindrosperompsin (CYN) from Enzo Life Sciences GmbH, Lausen, Switzerland; total RNA and cDNA High Capacity Archive Kit, TaqMan Universal PCR Master Mix and Taqman Gene Expression Assays were from Applied Biosystems, USA. All other chemicals were of analytical grade unless otherwise specified. A 0.5 mg/ml stock solution of CYN was prepared in 50% methanol.

2.2. Blood sampling and treatment

Whole blood samples were taken from a healthy female donor (age 35 years; non-smoker) who had not been exposed to ionizing radiation, vaccinated or treated with drugs for a year before blood sampling. The whole venous blood was collected under sterile conditions in heparinised vacutainer tubes (Becton Dickinson, NJ, USA) containing lithium heparin as anticoagulant.

The comet assay and the micronucleus assay were conducted on the whole blood, while cytotoxicity and gene expressions were performed on isolated human peripheral blood lymphocytes (HPBLs) cultivated at 37 °C in an atmosphere with 5% CO_2 (Heraeus Heracell 240 incubator, Langenselbold, Germany). HPBLs were isolated by Histopaque-1119 density gradient centrifugation, washed with RPMI medium and centrifuged at 4000 × *rpm* for 8 min. The pellet containing lymphocytes was used for further experiments. The isolated HPBLs were cultured in RPMI 1640 medium supplemented with 14% fetal calf serum and 9 mg/ml phytohemagglutinin.

The whole blood was treated with 0, 0.05, 0.1 and 0.5 μg/ml CYN for the comet assay and the cytokinesis-block micronucleus (CBMN) assay, while for the mRNA expression the isolated HPBLs were exposed to 0.5 μg/ml of CYN for 4 and 24 h. In each experiment, a non treated control and a vehicle control (0.5% methanol) were included.

2.3. Cell viability (cytotoxicity) test

Cell viability was evaluated by Trypan Blue exclusion method. HPBLs were isolated by Histopaque-1119 density gradient centrifugation. Treated and control cells were stained with trypan blue (0.4%). Viable (uncoloured) and dead (blue) cells were counted. The ratio of the number of viable cells/all cells gives the percentage of viable cells and the survival was normalized to solvent control. A total of 100 cells per repetition were examined with an Olympus AX-70 microscope (Tokyo, Japan).

2.4. The comet assay

The alkaline comet assay was carried out as described by Singh et al. (1988) with minor modifications (Gajski et al., 2008). Briefly, after the exposure to CYN, 5 μl of the whole blood was mixed with 100 μl of 0.5% LMP agarose and added to fully frosted slides pre-coated with 0.6% NMP agarose. After solidifying, the slides were covered with 0.5% LMP agarose, and the cells were lysed (2.5 M NaCl, 100 mM $EDTANa_2$, 10 mM Tris, 1% sodium sarcosinate, 1% Triton X-100, 10% dimethyl sulfoxide, pH10) overnight at 4 °C. After the lysis, the slides were placed into alkaline solution

(300 mM NaOH, 1 mM $EDTANa_2$, pH13) for 20 min at 4 °C to allow DNA unwinding and subsequently electrophoresed for 20 min at 1 V/cm. Finally, the slides were neutralized in 0.4 M Tris buffer (pH 7.5) for 5 min 3 times, stained with EtBr (20 μg/ml) and analyzed at 250× magnification using an epifluorescence microscope (Zeiss, Göttingen, Germany) connected through a black and white camera to an image analysis system (Comet Assay II; Perceptive Instruments Ltd., UK). The percent of tail DNA was used to measure the level of DNA damage and a total of 200 randomly captured nuclei were examined from each slide (100 nuclei from each duplicate slide) in three independent experiments. Each experiment was conducted on different day. The results are shown as box plots.

Hydrogen peroxide at final concentration 1 mM and 10 min treatment was used as a positive control.

2.5. Cytokinesis-block micronucleus (CBMN) assay

The micronucleus assay was performed following the guidelines detailed by Fenech and Morley, (Fenech and Morley, 1985) with minor modifications as described by Gajski et al. (Gajski et al., 2008). After the exposure (4 and 24 h) to CYN (0, 0.05, 0.1 and 0.5 μg/ml) the whole blood (500 μl) was incubated in an Euroclone medium (Chromosome kit P, Euroclone, Milano, Italy) at 37 °C in an atmosphere of 5% CO_2. Cytochalasin-B (Sigma) was added at a final concentration of 3 μg/ml 44 h after the culture was started. The cultures were harvested at 72 h. The lymphocytes were fixed in the methanol-acetic acid solution (3:1), air-dried and stained with 5% Giemsa solution (Sigma). All slides were randomized and coded prior to analysis. One thousand binuclear lymphocytes were analyzed under a light microscope (Olympus CX41, Tokyo, Japan) at 400× magnification. Micronuclei (MNi), nucleoplasmic bridges (NPBs) and cells with nuclear buds (NBUDs) were counted in 1000 binucleated cells (BNC) per experiment and were scored according to the HUMN project criteria published by Fenech (Fenech, 2000) in three independent experiments. In addition, the frequency of the binucleated cells with MNi, was counted, as in some cells more than one MN can be found.

The cytokinesis-block proliferation index (CPBI) was determined by scoring 1000 cells with one to four nuclei. The CPBI was calculated using the formula $[M_1 + 2M_2 + 3(M_3 + M_4)]/1000$, where M_1–M_4 represent the number of cells with one to four nuclei, respectively, and M3 and M4 are equally considered to be in their third cycle and is expressed as NDI (nuclear division index) (Fenech, 2006; Kirsch-Volders et al., 2003).

Bleomycin at final concentration 0.04 μg/ml and 2 h treatment was used as a positive control.

2.6. mRNA expression

After the exposure to CYN, lymphocytes were centrifuged at 3000 rpm for 10 min. The pellets were washed with 1x diethyl pyrocarbonate-phosphate buffer (DEPC-PBS) and again centrifuged at 3000 rpm for 10 min. Total RNA from the lymphocytes was isolated using TRIzol reagent, and cDNA synthesized using 1 μg of total RNA and cDNA High Capacity Archive Kit (Applied Biosystems, USA), according to the manufacturer's protocols. Gene expression of *CYP1A1, CYP1A2*, *P53*, *MDM2*, *GADD45α*, *CDKN1A, BAX, BCL-2, GCLC*, *GPX1*, *GSR*, *SOD1* and *CAT* was quantified using real-time quantitative PCR (ABI 7900 HT Sequence Detection System, Applied Biosystems, USA). TaqMan Universal PCR Master Mix and the following Taqman Gene Expression Assays were used (all from Applied Biosystems): *CYP1A1* (cytochrome P450, family 1, subfamily A, polypeptide 1), Hs00153120_m1; *CYP1A2* (cytochrome P450, family 1, subfamily A, polypeptide 2), Hs01070374_m1; *P53* (tumor protein p53), Hs00153349_m1; *MDM2* (Mdm2, 'transformed 3T3 cell double minute 2′, p53 binding protein gene), Hs00234753_m1; *GADD45α* ('growth arrest and DNA damage-inducible gene, alpha'), Hs00169255_m1; and *CDKN1A* ('cyclin-dependent kinase inhibitor 1A') Hs00355782_m1; *CAT* (catalase), Hs00937387_m1; *SOD1* (superoxide dismutase 1, soluble) Hs00166575_m1; *GCLC* (glutamate-cysteine ligase, catalytic subunit) Hs00155249_m1; *GSR* (glutathione reductase) Hs00167317_m1; *GPX1* (glutathione peroxidase 1) Hs01028922_g1; *BAX* (BCL2 associated X protein) Hs99999001_m1; *BCL-2* (B-cell CLL/lymphoma 2) Hs00608023_m1.

The amplification of *GAPDH* probe was performed as an internal control. The conditions for PCR were 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The data obtained from Taqman Gene Expression Assays were analyzed using the ΔΔCt algorithm. The expression levels of target mRNAs were normalized to the *GAPDH* mRNA level. Three independent experiments were performed each time in two parallels. Each experiment was conducted on different day.

2.7. Statistics

The statistical analyses were performed with GraphPad Prism 5 software. One-way analysis of variance (ANOVA, Kruskal–Wallis) was used to analyze the differences between the treatments within each experiment. Dunnett's test was used for multiple comparison versus the control; $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***) were considered as statistically significant. When calculating differences between samples for the micronucleus assay and the gene expression profiles, statistical significance between treated group and control was determined by Two-tailed Student *t*-test comparison of the mean. $P < 0.05$ was considered significant.

3. Results

The viability of HPBLs exposed to 0.05, 0.1 and 0.5 μg/ml of CYN for 4 and 24 h was not significantly affected (data not shown). Therefore, these concentrations were used in further experiments as it is very important to evaluate genotoxic potential at non-toxic concentrations to avoid false positive/negative results due to cytotoxicity and not genotoxicity.

3.1. Induction of DNA strand breaks

The whole blood was exposed to CYN (0, 0.05, 0.1 and 0.5 μg/ml) for 4 and 24 h, and the DNA damage in HPBLs

was determined with the comet assay. A statistically significant ($P < 0.05$) increase in the amount of DNA strand breaks was observed after 4-h exposure to 0.5 μg/ml CYN (Fig. 1A), while after 24 h slight increase of DNA damage was detected at 0.05 and 0.1 μg/ml (Fig. 1B).

3.2. Induction of micronuclei, nucleoplasmic bridges and nuclear buds

The genotoxic activity of CYN was further evaluated using the CBMN assay. The induction of micronuclei (MNi), nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) was assessed in binucleated lymphocytes (Fig. 2A–H).

The frequency of MNi (the number of total MNi detected in 1000 binucleated cells; Fig. 2A) and the number of cells containing MNi (Fig. 2C) statistically increased following 0.5 μg/ml CYN treatment at 4 h (Fig. 2A and C, respectively) and concentrations >0.1 μg/ml CYN at 24 h (Fig. 2B and D, respectively). Only slight induction of NPBs was observed. (Fig. 2E and F). The increase in the number of NBUDs after the exposure of HPBLs to CYN for 4 and 24 h (Fig. 2G and H) was detected at all tested concentrations, but was statistically significant only at 0.05 and 0.1 μg/ml after 4 h and at 0.1 μg/ml after 24 h (Fig. 2H).

After 24 h of exposure to CYN a statistically significant dose dependent decrease in NDI was observed (Fig. 3B).

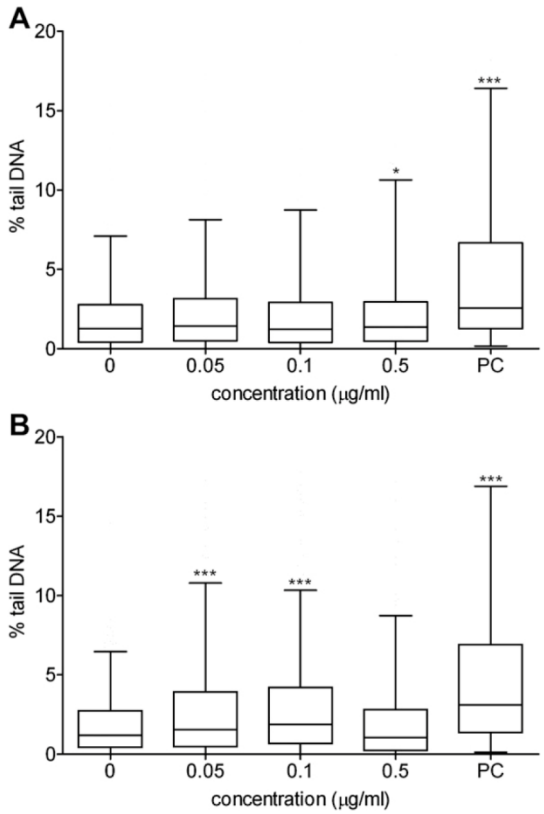

Fig. 1. DNA damage induced by CYN in HPBLs. The whole blood was exposed to 0, 0.05, 0.1 and 0.5 μg/ml CYN for 4 h (A) and 24 h (B) and then the comet assay was performed. DNA damage is expressed as percent of tail DNA. 200 cells were analyzed per experimental point in each of the three independent experiments. Data are presented as quantile box plots. The edges of the box represent the 25th and 75th percentiles, the mean values are represented as a solid line through the box, and the box plot bars represent the 95% confidence intervals. The asterix (*) denotes a significant difference between the vehicle control and CYN-treated cells [Kruskal–Wallis test; $P < 0.05$ (*) and $P < 0.001$ (***)]. Non-treated control cells did not differ significantly from solvent control cells (data not shown). Hydrogen peroxide (1 mM, 10 min) was used as a positive control (PC).

3.3. Effect of CYN on mRNA expression

The mRNA expression of selected genes was analyzed after 4 and 24-h exposure of HPBLs to 0.5 μg/ml of CYN by quantitative real-time PCR (Table 1).

3.3.1. Effect of CYN on the mRNA expression of CYP1A1 and CYP1A2

In HPBLs exposed to CYN for 4 h, the mRNA level of *CYP1A1* (2.274 ± 0.686) was significantly increased compared to solvent control, while the expression of mRNA level of *CYP1A2* could not be detected. After 24 h of exposure, both genes, *CYP1A1* (15.280 ± 9.267) and *CYP1A2* (7.829 ± 2.746), were significantly ($P < 0.05$) up-regulated.

3.3.2. Effect of CYN on the mRNA expression of DNA damage and apoptosis responsive genes

After the exposure of HPBLs to CYN for 4 h, the mRNA expressions of *P53*, *MDM2* and *GADD45α* were not affected, while they were up-regulated (1.603 ± 0.198, 1.554 ± 0.245 and 1.566 ± 0.372, respectively) after 24 h. The mRNA level of *CDKN1A* after the exposure to CYN was not changed at any exposure time compared to solvent control.

The mRNA levels of pro-apoptotic gene *BAX* and anti-apoptotic gene *BCL-2* were also not changed after 4 h exposure, while after 24 h the expressions of *BAX* (1.265 ± 0.172) and *BCL-2* (1.512 ± 0.157) were significantly up-regulated.

3.3.3. Effect of CYN on the mRNA expression of oxidative stress responsive genes

No changes in the expressions of selected oxidative stress responsive genes were observed in HPBLs exposed to CYN for 4 h. After 24 h exposure the expressions of *GPX1* (1.664 ± 0.315), *GSR* (1.544 ± 0.376), *GCLC* (2.314 ± 0.526) and *SOD1* (1.469 ± 0.422) were significantly higher than those of the solvent treated cells, while *CAT* (0.844 ± 0.211) was not changed compared to control.

4. Discussion

The present study provides the first evidence that CYN is genotoxic in human peripheral blood lymphocytes (HPBLs). For the first time it was shown that in HPBLs CYN induced micronuclei and nuclear bud formation and to a minor extent also nucleoplasmic bridges and DNA strand breaks. At the molecular level the toxin induced up-regulation of cytochrome P450 metabolic enzymes (*CYP1A1* and *CYP1A2*), providing further evidence that these enzymes are likely to be involved in metabolic activation of CYN. The up-regulation of *P53* and its downstream regulated DNA damage marker genes (*MDM2*, *GADD45α*) as well as oxidative stress (*GPX1*,

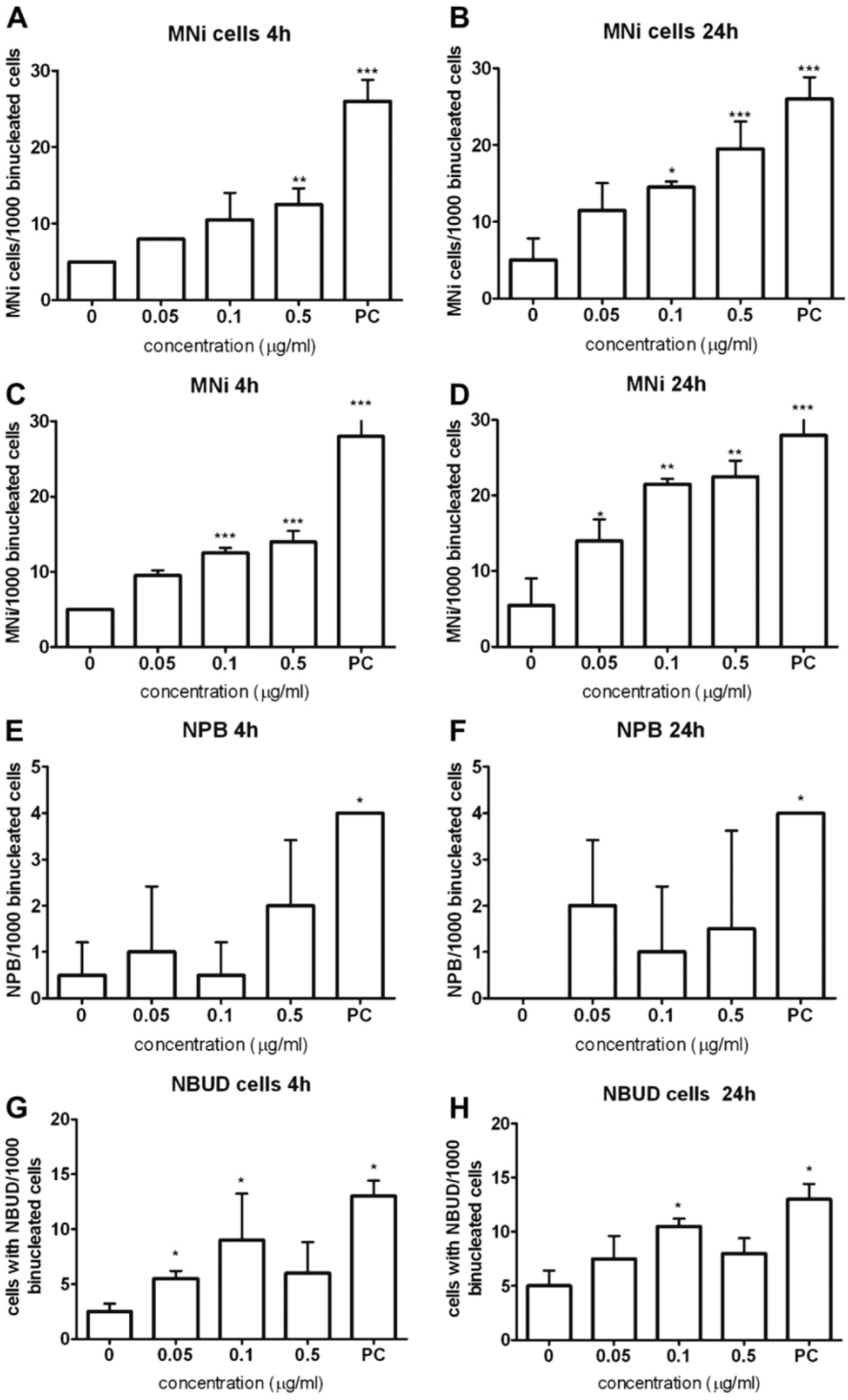

Fig. 2. Number of binucleated cells with A–B) micronucleated cells (MN cells), C–D) micronuclei (MNi), E–F) nuclear bridges (NPBs) and G–H) cells with nuclear buds (NBUDs) per 1000 binucleated peripheral blood lymphocytes after the exposure to CYN (0, 0.05, 0.1 and 0.5 μg/ml) for 4 h (A, C, E and G) and 24 h (B, D, F and H). The number of MN cells shows how many cells contained MN, while MNi shows the overall number of MN as some cells can possess more than one MN. Statistical significance between treated groups and the control was determined by two tailed Student's *t*-test and $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***) were considered as statistically significant. Bleomycin (0.04 μg/ml, 2 h) was used as a positive control (PC).

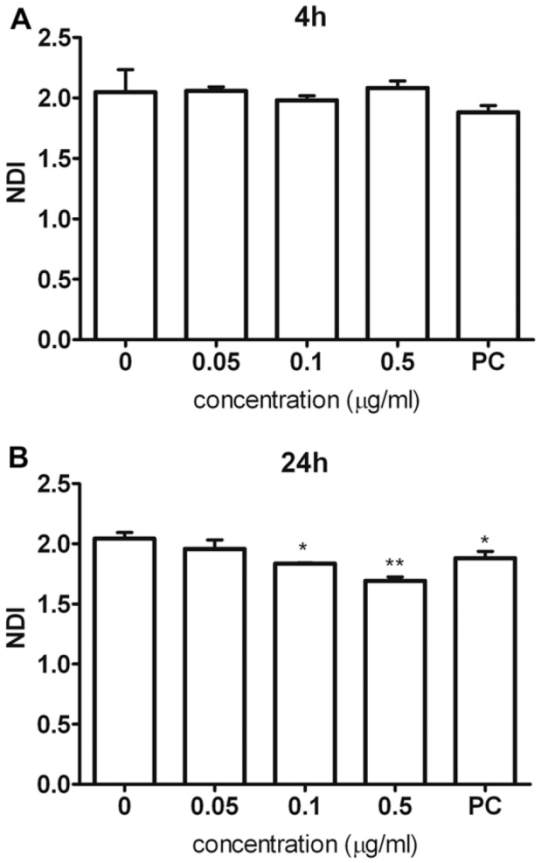

Fig. 3. The influence of CYN (0, 0.05, 0.1 and 0.5 μg/ml) on nuclear division index (NDI) after A) 4 and B) 24-h exposure. Statistical significance between treated groups and the control was determined by two tailed Student's *t*-test and $P < 0.05$ (*). Bleomycin (0.04 μg/ml, 2 h) was used as a positive control (PC).

GSR, *GCLC*, *SOD1*) responsive genes and apoptotic genes (*BAX*, *BCL-2*) was shown. To our knowledge this is new information as the expression of these genes in HPBLs was not studied before. In previously published studies concentrations of CYN up to 2 μg/ml in hepatic cells (Bain et al., 2007; Bazin et al., 2010a; Humpage et al., 2005; Straser et al., in press) and 10 μg/ml in non-hepatic cells (Fessard and Bernard, 2003; Humpage et al., 2000; Lankoff et al., 2007) were used and the exposure of populations to such high concentrations of cylindrospermopsin is not likely. In our study genotoxic potential of CYN in non-hepatic cells was shown at relatively low concentrations (0.5 μg/ml). In the environment CYN was reported at concentrations in the range of 0.16–1.8 μg/l (Kokociński et al., 2009), while in pond water it can reach the concentrations up to 0.6 mg/l (Saker and Eaglesham, 1999; Shaw et al., 2000). CYN has previously been shown to be genotoxic *in vitro* (Bazin et al., 2010a; Humpage et al., 2000, 2005; Straser et al., in press) and *in vivo* (Bazin et al., 2010b; Shaw et al., 2000; Shen et al., 2002) and that metabolic activation is required for its genotoxic effects. The later was confirmed using the cytochrome P450 inhibitors (Bazin et al., 2010a; Humpage et al., 2005). In

Table 1
Changes in gene expression of *CYP1A1*, *CYP1A2*, *P53*, *MDM2*, *GADD45α*, *CDKN1A*, *BAX*, *BCL-2*, *GPX1*, *GSR*, *GCLC*, *SOD1* and *CAT* after the exposure of HPBLs to CYN (0 and 0.5 μg/ml) for 4 and 24 h. The results are expressed as relative mRNA expression normalized to solvent control ± STD. (*) Denotes a significant difference between CYN-treated groups and control (Student's *t*-test; $P < 0.05$).

gene	CYN conc. (μg/ml)	4 h	24 h
CYP1A1	0	1.124 ± 0.608	1.011 ± 0.167
	0.5	2.274 ± 0.686	15.28 ± 9.267*
CYP1A2	0	n.d.	1.069 ± 0.434
	0.5	n.d.	7.829 ± 2.746*
P53	0	1.006 ± 0.121	1.001 ± 0.037
	0.5	0.909 ± 0.114	1.603 ± 0.198*
MDM2	0	1.001 ± 0.045	1.005 ± 0.109
	0.5	1.117 ± 0.091	1.554 ± 0.245*
CDKN1A	0	1.000 ± 0.031	1.002 ± 0.076
	0.5	1.121 ± 0.053	0.992 ± 0.050
GADD45a	0	1.000 ± 0.014	1.005 ± 0.112
	0.5	1.141 ± 0.127	1.566 ± 0.372*
BAX	0	1.000 ± 0.034	1.000 ± 0.024
	0.5	1.062 ± 0.038	1.265 ± 0.172*
BCL-2	0	1.002 ± 0.063	1.000 ± 0.013
	0.5	1.094 ± 0.120	1.512 ± 0.157*
GCLC	0	1.000 ± 0.029	1.002 ± 0.073
	0.5	0.987 ± 0.059	2.041 ± 0.587*
GSR	0	1.001 ± 0.041	1.000 ± 0.035
	0.5	1.076 ± 0.059	1.415 ± 0.355*
GPX1	0	1.001 ± 0.035	1.002 ± 0.069
	0.5	1.086 ± 0.173	1.664 ± 0.315*
SOD1	0	1.007 ± 0.134	1.003 ± 0.080
	0.5	1.169 ± 0.231	1.397 ± 0.364*
CAT	0	1.001 ± 0.040	1.001 ± 0.043
	0.5	1.103 ± 0.101	0.844 ± 0.211

n.d.- not detected.

the present study we showed that in HPBLs non-toxic concentrations of CYN induced significant increase of DNA strand breaks after 4 and 24 h of exposure. Similarly, Humpage et al. (2005) and Straser et al. (in press) reported the induction of DNA damage in primary mouse hepatocytes and HepG2 cells, respectively. On the contrary, Fessard and Bernard (Fessard and Bernard, 2003) failed to show the induction of strand breaks in metabolically incompetent CHO-K1 cells. Further, no chromosomal aberrations were detected in CHO-K1 cells regardless the presence of S9 metabolic activation (Lankoff et al., 2007), while at higher applied concentrations CYN affected the microtubular structure in CHO-K1 cells, potentially leading to disruption of spindle or centromere function and leading to the loss of whole chromosomes (Gácsi et al., 2009).

It is generally accepted that toxicity and genotoxicity of CYN is CYP-450–metabolism dependent (Bazin et al., 2010a Froscio et al., 2003; Humpage et al., 2005; Norris et al., 2002; Runnegar et al., 1995; Straser et al., in press). In HPBLs we detected a dose and time dependent increase in the formation of MNi and MNed cells. CYN induced MNi formation has been previously reported in four human cell lines; the lymphoblastoid cell line, WIL2–NS (Humpage et al., 2000), liver-derived HepaRG cells, colon-derived Caco-2 cells (Bazin et al., 2010a) and human hepatoma HepG2 cells (Straser et al., in press). CYN induced DNA fragmentation and loss of whole chromosomes in the WIL2–NS lymphoblastoid cell line, indicating its clastogenic and aneugenic activity (Humpage et al., 2000). In differentiated HepaRG cells, which

express metabolic enzymes at levels comparable to those in primary human hepatocytes (Jossé et al., 2008; Kanebratt and Andersson, 2008), significant increase in MNi formation was detected (Bazin et al., 2010a), while in undifferentiated HepaRG cells, which have lower CYP expression (Kanebratt and Andersson, 2008), no induction of MNi following CYN exposure was observed (Bazin et al., 2010a). Cytokinesis-block micronucleus (CBMN) assay is the preferred method for measuring micronuclei (MNi); however, new developments indicate that it can also be used for measuring nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs). NPBs provide a measure of DNA mis-repair, chromosome rearrangement or telomere end-fusion and are assumed to occur when the centromeres of dicentric chromosomes are pulled to opposite poles of the cell at anaphase. In the process of nuclear budding, which occurs during S-phase, amplified and/or excess DNA is removed from the nucleus and is a marker of gene amplification (Fenech, 2006). In HPBLs minor increase in NPBs formation was detected after CYN exposure, while there was a significant induction of NBUDs. To our knowledge our study is the first, where the effect of CYN on the formation of NPBs and NBUDs in HPBLs had been investigated. Recently, Straser et al. (in press) reported that CYN significantly increased the formation of MNi, NBUDs and NPBs in HepG2 cells. These results indicate that CYN induces complex genomic alterations including structural chromosomal rearrangements and gene amplification.

In recent years toxicogenomic analysis has become widely used as a tool for hazard identification and risk assessment of genotoxic and carcinogenic properties of different substances especially in combination with classical genotoxicity assays and endpoints (Ellinger-Ziegelbauer et al., 2009). Studies on the modulations of gene expression after CYN exposure are limited. In this respect we evaluated the influence of CYN (0.5 μg/ml) on the alteration of mRNA levels of various genes leading to the activation of pathways involved in the metabolism, response to DNA damage and repair, oxidative stress, survival and/or cell death.

There is evidence that CYP-450 family enzymes are involved in metabolic activation of CYN; however, it is not clear which isoforms are involved. Broad-spectrum monooxigenase inhibitor SKF525A and omeprazole completely prevented CYN induced DNA strand breaks (Humpage et al., 2005), while ketoconazole, a potent competitive CYP3A4 inhibitor, attenuated CYN induced MNi formation in Caco-2, but not in HepaRG cells (Bazin et al., 2010a). Exclusive role of CYP3A4 in the metabolic activation of CYN could not be confirmed (Bazin et al., 2010a) as ketoconazole is also a potent inhibitor of CYP1A1 and a moderate inhibitor of CYP1A2, CYP2C and CYP 2D6 (Pelkonen et al., 2008).

From the literature it is known that human lymphocytes possess cytochrome P450 (CYP450) enzymes (Garte et al., 2003) such as CYP1A1 (Garte et al., 2003) and CYP1A2 (Haas et al., 2005). In HPBLs time and dose dependent up-regulations of *CYP1A1* and *CYP1A2* were detected, which were approximately 15-fold and 8-fold increased after 24 h exposure, respectively. The CYP1A1 and 1A2 isoforms are highly inducible enzymes, widely known as activators of numerous carcinogenic compounds, and here we provide the first evidence that exposure to CYN induced transcription of these two enzymes, supporting previous assumptions that they are involved in CYN metabolic activation to genotoxic intermediate(s). However, with these results we can not exclude possible involvement of other CYP isoforms in the metabolic activation of CYN.

We further analyzed the changes in the expression of P53-downstream regulated genes (*CDKN1A*, *MDM2*, *GADD45α*, *BAX*, *BCL-2*). The tumour-suppressor gene, *P53*, plays the central role in DNA damage response pathways and cancer development (Vogelstein et al., 2000) by activating its target genes involved in the accelerated DNA repair, inhibition of cell cycle progression, senescence, differentiation and apoptosis (Oren, 2003). The negative regulation of P53, under non-stressed conditions, is performed by the Mdm2 protein, which is a product of a proto-oncogene, amplified or otherwise over-expressed in a significant number of human tumours (Michael and Oren, 2002). The main cellular event regulated by P53 is the cell cycle control. The P53 protein directly stimulates the expression of P21WAF1/CIP1, an inhibitor of cyclin-dependent kinases (CDKs), which results in G1/S arrest (Vogelstein et al., 2000). P53 is also able to control G2/M arrest via GADD45 (Smith et al., 1994). *Gadd45a* gene can be induced by DNA damaging agents and growth arrest signals (Zhan, 2005). The results from the present study showed that after 24 h of exposure of HPBLs to CYN a significant up-regulation of the *P53* gene as well as its downstream regulated genes, *MDM2* and *GADD45α*, was detected, while there were no differences in the *CDKN1A* mRNA levels compared to control cells. As the gene expressions were measured at only two time points (4 and 24 h) it is possible that *CDK1A* was induced later than other genes. Similarly Laffon et al. (2001) showed that exposure of human peripheral lymphocytes to styrene-7.8-oxide resulted in up-regulation of *P53* mRNA and subsequent up-regulation of *CDK1A* mRNA after longer exposure. In addition, in the same study the authors also showed that there could be differences in responses at the level of DNA damage responsive genes between different donors Laffon et al. (2001).

Apoptosis, programmed cell death, is another important cell process regulated by P53 (Perfettini et al., 2004). The gene expression profile of *BAX* and *BCL-2*, after 24 h exposure of HPBLs to CYN showed a significant up-regulation of both genes with *BCL-2* being more pronounced. Concurrent up-regulation of both genes has been reported in numerous studies; however, it is stated that it is the ratio of BAX/BCL-2 that is crucial for determination of sensitivity to apoptosis (Laffon et al., 2001; Woo et al., 2000). From our results it can be seen that the ratio is in favour of *BCL2*, indicating the suppression of apoptosis at applied concentration (0.5 μg/ml). To date limited data has been published on the induction of apoptosis by CYN. In CHO-K1 cells, CYN induced apoptosis at low doses (1–4 μM) and prolonged incubation time (Gácsi et al., 2009; Lankoff et al., 2007). There are only two publications found in the literature, where the influence of CYN on the gene expression was studied. Increased expression of P53 target genes (*CDKN1A*, *GADD45α*, *BAX* and *MDM2*) was detected in human dermal fibroblasts (HDFs) (Bain et al., 2007) and human hepatoma cells (HepG2) (Bain et al., 2007; Straser et al., in press).

It was described that one mechanism of CYN toxicity is the inhibition of glutathione synthesis (Humpage et al., 2005; Runnegar et al., 1995), which can lead to increased

oxidative stress and consequently genotoxicity. Glutathione (GSH) plays the central role in the cellular defence against reactive species and in signaling pathways, it is important in maintaining the cellular homeostasis as well as in the detoxification of a variety of xenobiotics (Boyland and Chasseaud, 1969). In the presence of GSH, glutathione peroxidases (GPXs) catalyze the reduction of organic hydroperoxides or hydrogen peroxide and thereby glutathione disulfide (GSSG) is formed (Knapen et al., 1999). The formation of GSSG or GSH-conjugates can result in the depletion of cellular GSH, which can be opposed by two major routes; through reduction of the GSSG via glutathione reductase (GSR) and through *de novo* synthesis in a so called γ-glutamyl cycle (Meister and Anderson, 1983). The *de novo* biosynthesis of GSH requires two ATP-dependent enzymes glutamate–cysteine ligase (GCL) and glutathione synthase (GS) (Griffith, 1999).

To date, there is no published report concerning possible changes in the expression of genes involved in oxidative stress in cells exposed to CYN. The results obtained on HPBLs revealed that the gene expressions of both enzymes involved in maintaining GSH redox homeostasis, *GPX1* and *GSR,* were significantly up-regulated after 24-h exposure of cells to CYN, indicating the oxidative stress induction by CYN. As already mentioned, CYN was reported to inhibit glutathione synthesis (Humpage et al., 2005; Runnegar et al., 1995). However, this assumption was made mostly from the measurements of GSH levels after the exposure of cells to CYN. Decreased GSH content in the cells could result from increased GSH efflux, decreased GSH synthesis, increased oxidation of GSH or formation of GSH-conjugates, or a combination of these. No data exist on direct measurement of CYN-GSH conjugate formation. In a study on mice exposed to CYN Norris et al. (2002) calculated whether the reduction of hepatic GSH could be due to the formation of conjugates CYN-GSH. Their calculation showed that conjugate formation could not deplete liver glutathione to the extent of measured depletion and they suggested that the reason was the inhibition of GSH synthesis. In the present study the up-regulation of *GCLC* by more than 2-fold, was determined, showing a possible increase in the biosynthesis of GSH. The up-regulation of *GCLC* is a response of the cells to a depletion of GSH but this does not mean the final increase in biosynthesis of GSH as it can be inhibited in later steps. It is known that protein synthesis is not regulated only at the transcriptional, but also at the posttranscriptional level by changes in the mRNA stability, mRNA translation regulation, and at the level of posttranslational modifications.

The primary antioxidant enzymes in cells are superoxide dismutases (SODs) and catalase (Fridovich, 1978). The gene expression profile of these two antioxidant enzymes, after 24 h exposure of HPBLs to CYN, showed slight, though, significant up-regulation of *SOD1*, while *CAT* was not changed. As these two enzymes are usually up-regulated by severe oxidative stress, these results suggest that CYN induced only minor oxidative stress; however the mechanism of oxidative stress induction by CYN has to be elucidated in more details.

Based on these results we can conclude that CYN is genotoxic and not only the liver and colon cells, but also lymphocytes can be a target of CYN induced genotoxicity. Our results indicated the induction of DNA damage in non-liver cells and changes in the mRNA expression of metabolic enzymes *CYP1A1* and *CYP1A2*, DNA damage responsive genes *P53*, *GADD45α* and *MDM2*, genes involved in the antioxidant defence *GPX*, *GSR*, *GCLC* and *SOD1*, as well as apoptosis genes *BAX* and *BCL-2*. However, there is still a need to further confirm these results on the protein level, which will help to clarify the mechanisms involved in CYN genotoxic activity.

Conflict of interest

The authors report no conflict of interest.

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2.1.3 Mikrocistin-LR povzroča poškodbe DNK pri humanih perifernih limfocitih

Microcystin-LR induced DNA damage in human peripheral blood lymphocytes

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Zaradi vse pogostejšega pojavljanja cianobakterijskih cvetenj, ki so posledica globalnega segrevanja in naraščajoče evtrofikacije voda, je izpostavljenost ljudi mikrocistinom, ki jih proizvajajo sladkovodne vrste cianobakterij, vse bolj zaskrbljujoče. Čeprav so mikrocistini znani kot hepatotoksini, lahko vplivajo tudi na druga tkiva. Dokazano je bilo, da ti toksini povzročajo poškodbe DNK *in vitro* in *in vivo*, vendar so mehanizmi njihove genotoksične aktivnosti še vedno nejasni. Pri humanih limfocitih iz periferne krvi (HPBL) so necitotoksične koncentracije (0, 0,1, 1 in 10 µg/ml) mikrocistina-LR (MCLR) povzročile od koncentracije in časa odvisno povečanje poškodb DNK, ki smo jih merili s testom komet. Po razgradnji DNK izoliranih HPBL, ki so bili izpostavljeni MCLR, s formamidopirimidin glikozilazo (Fpg), smo zaznali večje število prelomov DNK verig kot pri neencimsko razgrajeni DNK, kar potrjuje, da MCLR povzroča oksidativne poškodbe DNK. S testom mikrojeder nismo zaznali statistično značilnega povišanja MNi, NBUD ali NPB po 24 h izpostavljenosti HPBL MCLR. Prav tako nismo opazili sprememb na molekularnem nivoju, v izražanju izbranih genov, ki sodelujejo v celičnem odzivu na poškodbe DNK in oksidativni stres po 4 h izpostavljenosti MCLR (1 µg/ml). Po 24 h pa je prišlo do povišanega izražanja genov vpletenih v odziv na DNK poškodbe (*P53*, *MDM2*, *GADD45A*, *CDKN1A*), oksidativni stres (*CAT*, *GPX1*, *SOD1*, *GSR*, *GCLC*) in gena, vključenega v apoptozo (*BAX*). Ti rezultati podajajo dokaze, da je MCLR posredno genotoksičen agens, ki deluje preko sprožitve oksidativnega stresa, in da so tudi limfociti tarča toksičnih učinkov MCLR.

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Microcystin-LR induced DNA damage in human peripheral blood lymphocytes

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Gene expression

ABSTRACT

Human exposure to microcystins, which are produced by freshwater cyanobacterial species, is of growing concern due to increasing appearance of cyanobacterial blooms as a consequence of global warming and increasing water eutrophication. Although microcystins are considered to be liver-specific, there is evidence that they may also affect other tissues. These substances have been shown to induce DNA damage *in vitro* and *in vivo*, but the mechanisms of their genotoxic activity remain unclear. In human peripheral blood lymphocytes (HPBLs) exposure to non-cytotoxic concentrations (0, 0.1, 1 and 10 μg/ml) of microcystin-LR (MCLR) induced a dose- and time-dependent increase in DNA damage, as measured with the comet assay. Digestion of DNA from MCLR-treated HPBLs with purified formamidopyrimidine-DNA glycosylase (Fpg) displayed a greater number of DNA strand-breaks than non-digested DNA, confirming the evidence that MCLR induces oxidative DNA damage. With the cytokinesis-block micronucleus assay no statistically significant induction of micronuclei, nucleoplasmic bridges and nuclear buds was observed after a 24-h exposure to MCLR. At the molecular level, no changes in the expression of selected genes involved in the cellular response to DNA damage and oxidative stress were observed after a 4-h exposure to MCLR (1 μg/ml). After 24 h, DNA damage-responsive genes (*p53*, *mdm2*, *gadd45a*, *cdkn1a*), a gene involved in apoptosis (*bax*) and oxidative stress-responsive genes (*cat*, *gpx1*, *sod1*, *gsr*, *gclc*) were up-regulated. These results provide strong support that MCLR is an indirectly genotoxic agent, acting via induction of oxidative stress, and that lymphocytes are also the target of microcystin-induced toxicity.

1. Introduction

Cyanobacteria are well known to produce a variety of toxins, which are released into the environment after cell death. Microcystins (MCs) are the largest group of cyanobacterial toxins consisting of over 80 isoforms, microcystin-LR (MCLR) being the most common and most widely studied congener [1]. Based on acute toxicity studies it is considered as one of the most potent cyanobacterial toxins. In 1998 the World Health Organization (WHO) established a provisional guideline value for MCLR of 1 μg/l in drinking water [2] and recently the International Agency for Research on Cancer (IARC) classified MCLR as a possible human carcinogen (Group 2B) [3].

MCs are highly hepatotoxic and one of the mechanisms of their toxicity is the inhibition of eukaryotic serine/threonine protein phosphatases 1 and 2A (PP1 and PP2A) [4–6] and consequently hyperphosphorylation of cytoskeletal proteins [6]. This leads to disruption of many cellular processes and cytoskeletal damage [7,8]. More importantly, these toxins are potent tumour promoters [9–11]. The tumour-promoting activity of MCLR is supposed to arise from its ability to inhibit PP2A, which regulates several mitogen-activated kinases (MAPK) and these in turn regulate transcription of genes required for cell proliferation [12]. Apart from the tumour-promoting activity there is some evidence that MCs can act as tumour initiators [13]. There is also increasing evidence that MCs are genotoxic [14–20], but the mechanisms by which these toxins induce DNA damage and cancer development are not well understood. Moreover, existing data on their genotoxic potential are contradictory.

Human exposure to MCs is of growing concern due to increasing appearance of cyanobacterial blooms as a consequence of water eutrophication and global warming. The main routes of human exposure are during recreational activity, through drinking of water contaminated with cyanotoxins and by ingestion of food and dietary supplements that may be contaminated with cyanobacteria [1,21–23]. A minor route of exposure to cyanotoxins is the parenteral route through haemodialysis, which nevertheless represents an extremely relevant route of exposure as it results in a high internal dose of the toxins, directly entering the bloodstream [24,25].

For a long time the liver was thought to be the main target organ of MC activities, as these toxins require uptake via active transport by organic anion-transporting polypeptides (OATP), which are

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expressed in the hepatocytes [26]. OATPs are now being increasingly recognized to be expressed not only in the liver but also in the gastrointestinal tract, kidney, and brain [27]. There is evidence that MCLR can be transported across the human blood–brain barrier [26]. When orally ingested, MCs are transported across the ileum into the bloodstream through the bile-acid transport system present in hepatocytes and in cells lining the small intestine [28].

The aim of the present study was to evaluate the induction of DNA strand-breaks and micronuclei (MNi) in human peripheral blood lymphocytes (HPBLs) after exposure to MCLR. In addition, the mechanism of MCLR-induced genotoxicity has been evaluated by use of quantitative real-time-PCR, by examining – with quantitative real-time-PCR – the gene-expression pattern of selected DNA damage-responsive genes and genes involved in oxidative stress and apoptosis.

2. Materials and methods

2.1. Chemicals

Chromosome kit P was from Euroclone, Italy; fetal bovine serum, RPMI 1640, and TRIzol reagent were from Invitrogen, Carlsbad, USA; penicillin/streptomycin, cytochalasin B, histopaque, ethidium bromide, low melting-point (LMP) and normal melting-point (NMP) agaroses were from Sigma, USA; phytohaemagglutinin was from Remel Europe Ltd., England; heparinised vacutainer tubes from Becton Dickinson, USA; Giemsa from Merck, Germany; microcystin-LR from Enzo Life Sciences GmbH, Lausen, Switzerland; Fpg FLARE™ assay kit from Trevigen Inc., USA; total RNA and cDNA High-Capacity Archive Kit, TaqMan Universal PCR Master Mix and Taqman Gene Expression Assays were from Applied Biosystems, USA. All other chemicals were of analytical grade.

2.2. Blood sampling and treatment

Whole blood samples were taken from a healthy male donor (age 28 years; non-smoker) who had not been exposed to ionizing radiation, vaccinated or treated with drugs for a year before blood sampling. Whole venous blood was collected under sterile conditions in heparinised vacutainer tubes (Becton Dickinson, NJ, USA) containing lithium heparin as anticoagulant.

The comet assay and the micronucleus assay were conducted on whole blood, while cytotoxicity and gene expressions were performed on isolated human peripheral blood lymphocytes (HPBLs) cultivated at 37 °C in an atmosphere with 5% CO_2 (Heraeus Heracell 240 incubator, Langenselbold, Germany). HPBLs were isolated by Histopaque-1119 density gradient centrifugation, washed with RPMI medium and centrifuged at 4000 × rpm [give proper "2880 × *g*" value here; Ed.] for 8 min. The pellet of lymphocytes was used for further experiments. The isolated HPBLs were cultured in RPMI-1640 medium supplemented with 14% fetal calf serum and 9 mg/ml phytohaemagglutinin.

The whole blood was treated with 0, 0.1, 1 and 10 μg/ml MCLR for 4, 6 and 24 h for the comet assay and during 24 h for the cytokinesis-block micronucleus (CBMN) assay, while for the mRNA expression the isolated HPBLs were exposed to 1 μg/ml of MCLR for 4 and 24 h. In each experiment a non-treated control and a vehicle control (0.1% ethanol) were included.

2.3. Cell-viability (cytotoxicity) test

Indices of cell viability were established by differential staining of HPBLs with acridine orange and ethidium bromide [29]. A total of 100 cells per replicate were examined with a Zeiss microscope (Göttingen, Germany). Cells were classified as follows: live cells with functional membrane, with uniform green staining of the nucleus, and necrotic cells with uniform orange staining of the nucleus.

2.4. The comet assay

The alkaline comet assay was carried out as described by Singh et al. [30] with minor modifications [29]. Briefly, after the exposure to MCLR, 5 μl of whole blood was mixed with 100 μl of 0.5% LMP agarose and added to fully frosted slides precoated with 0.6% NMP agarose. After solidification, the slides were covered with 0.5% LMP agarose, and lysed (2.5 M NaCl, 100 mM EDTA-Na_2, 10 mM Tris, 1% sodium sarcosinate, 1% Triton X-100, 10% dimethyl sulfoxide, pH10) overnight at 4 °C. After the lysis, the slides were placed in alkaline solution (300 mM NaOH, 1 mM EDTA-Na_2, pH13) for 20 min at 4 °C to allow DNA unwinding, and subsequently electrophoresed for 20 min at 1 V/cm. Finally, the slides were neutralized in 0.4 M Tris buffer (pH 7.5) – three times 5 min – stained with EtBr (20 μg/ml) and analyzed at 250 × magnification using an epifluorescence microscope (Zeiss, Göttingen, Germany) connected through a black-and-white camera to an image-analysis system (Comet Assay II; Perceptive Instruments Ltd., UK). The percent of tail DNA was used to measure the level of DNA damage and a total of 50 randomly captured nuclei were examined from each slide in two independent experiments. The results are shown as box plots.

The analysis of the formation of oxidized purines was performed with the Fpg FLARE™ assay kit (Trevigen Inc., Gaithersburg, USA) with minor modifications. The slides were prepared as described above. For each sample and control, 5 μl of whole blood was mixed with 100 μl of LMP agarose (provided with the FLARE™ assay kit) and placed on the slides. After solidification the slides were covered with 0.5% LMP agarose and then immersed in a pre-chilled lysis solution (provided with the FLARE™ assay kit) and lysed overnight at 4 °C. After lysis, the slides were treated with 100 μl of Fpg enzyme freshly diluted in REC dilution buffer (1:500) or with 100 μl of REC dilution buffer only (control), covered with a cover glass and incubated at 37 °C for 30 min. Subsequently, the slides were immersed in an alkali solution (0.3 M NaOH, 1 mM Na_2EDTA; pH 12.1) for 40 min and electrophoresed for 20 min at 1 V/cm. Finally, the slides were neutralized, stained with EtBr and the comets were analyzed as described above.

2.5. Cytokinesis-block micronucleus (CBMN) assay

The micronucleus assay was performed according to the guidelines by Fenech and Morley [31] with minor modifications [29]. After the exposure (24 h) to MCLR the whole blood (500 μl) was incubated in a Euroclone medium (Chromosome kit P) at 37 °C in an atmosphere of 5% CO_2. Cytochalasin-B was added at a final concentration of 3 μg/ml, 44 h after the culture was started. The cultures were harvested after 72 h. The lymphocytes were fixed in methanol–acetic acid (3:1), air-dried and stained with 5% Giemsa solution (Sigma). All slides were randomised and coded prior to analysis. The binuclear lymphocytes were analyzed under a light microscope (Olympus CX41, Tokyo, Japan) at 400 × magnification. Micronuclei (MNi), nucleoplasmic bridges (NPB) and nuclear buds (NBUD) were counted in 1000 binucleated cells (BNC) per experimental point and were scored according to the HUMN project criteria published by Fenech [32]. Two independent experiments were performed. The cytokinesis-block proliferation index (CPBI) was determined by scoring 1000 cells with one to four nuclei. The CPBI was calculated using the formula $[M_1 + 2M_2 + 3(M_3 + M_4)]/1000$, where M_1–M_4 represent the number of cells with one to four nuclei, respectively, and M_3 and M_4 are equally considered to be in their third cycle [33,34]. Bleomycin at final concentration 0.04 μg/ml and 2 h treatment was used as a positive control.

2.6. mRNA expression

After the exposure to MCLR, the HPBLs were centrifuged at 3000 rpm for 10 min. The pellets were washed with 1 × DEPC-PBS and again centrifuged at 3000 rpm for 10 min. Total RNA from the lymphocytes was isolated using TRIzol reagent, and cDNA synthesized using 1 μg of total RNA and cDNA High-Capacity Archive Kit (Applied Biosystems, USA), according to the manufacturer's protocol. Gene expression of *p53*, *mdm2*, *gadd45α*, *cdkn1a*, *bax*, *bcl*, *cat*, *gpx1*, *gclc*, *sod1* and *gsr* was quantified using real-time quantitative PCR (ABI 7900 HT Sequence Detection System, Applied Biosystems, USA). TaqMan Universal PCR Master Mix and the following Taqman Gene Expression Assays were used (all from Applied Biosystems): *p53* (tumour protein p53), Hs00153349_m1; *mdm2* (Mdm2, 'transformed 3T3 cell double minute 2', p53 binding protein gene), Hs00234753_m1; *gadd45α* ('growth arrest and DNA damage-inducible gene, alpha'), Hs00169255_m1; and *cdkn1a* ('cyclin-dependent kinase inhibitor 1A'), Hs00355782_m1; *cat* (catalase), Hs00937387_m1; *sod1* (superoxide dismutase 1, soluble), Hs00166575_m1; *gclc* (glutamate-cysteine ligase, catalytic subunit), Hs00155249_m1; *gsr* (glutathione reductase), Hs00167317_m1; *gpx1* (glutathione peroxidase 1), Hs01028922_g1; *bax* (BCL2 associated X protein), Hs99999001_m1; *bcl2* (B-cell CLL/lymphoma 2), Hs00608023_m1.

Amplification of the *GAPDH* probe was performed as an internal control. The conditions for PCR were 50 °C for 2 min, 95 °C for 10 min and 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The data obtained from Taqman Gene Expression Assays were analyzed using the ΔΔCt algorithm. The expression levels of target mRNAs were normalized to the GAPDH mRNA level. Two independent experiments were performed, each time in two parallels.

2.7. Statistics

The statistical analyses were performed with GraphPad Prism 5 software. For the comet assay, one-way analysis of variance (ANOVA) was used to analyze the differences between treatments within each experiment. Dunnett's test was used for multiple comparison versus the control; $P < 0.05$ was considered as statistically significant (*). Difference between samples in the micronucleus test and – for the gene expression assays – the statistical significance of the difference between treated groups and controls was determined by Two-tailed Student's *t*-test comparison of the mean; $P < 0.05$ was considered significant.

3. Results

The viability of HPBLs exposed to 0.1, 1 and 10 μg/ml of MCLR for 4, 6 and 24 h was not significantly affected (data not shown). Therefore, these concentrations were used in further experiments.

3.1. Induction of DNA strand-breaks and Fpg-sensitive sites

Whole blood was exposed to MCLR (0.1, 1 and 10 μg/ml) for 4, 6 and 24 h, and the DNA damage in HPBLs was determined with the comet assay. Statistically significant ($P < 0.05$) increase of DNA damage was observed after a 6-h exposure to 1 and 10 μg/ml MCLR (Fig. 1B), while after 24 h an increase of DNA damage was detected at all test concentrations (Fig. 1C).

The induction of oxidative DNA damage was determined with the modified comet assay including the purified DNA damage-specific enzyme, Fpg, which recognizes and excises oxidized purines. Thus, the difference in the % tail DNA in enzyme-digested nuclei compared to non-digested (buffer-treated) reflects the amount of oxidized purines. In HPBLs exposed to MCLR for 4 h, no oxidation of purines was observed (Fig. 2A), while after 6 h a higher level of DNA strand-breaks after digestion with Fpg was detected at 10 μg/ml of MCLR (Fig. 2B). The maximal level of oxidized DNA bases was detected after 24 h and was dose-dependent (Fig. 2C).

3.2. Induction of micronuclei, nucleoplasmic bridges and nuclear buds

The genotoxic activity of MCLR was further evaluated by use of the CBMN assay. Following MCLR treatment during 24 h, no induction of MN, NBUDs and NPBs was observed in binucleated lymphocytes (Table 1).

3.3. Effect of MCLR on mRNA expression

The mRNA expression of selected genes was analyzed after 4 and 24 h of exposure of isolated HPBLs to 1 μg/ml of MCLR by quantitative real-time PCR (Table 2).

3.3.1. Effect of MCLR on the mRNA expression of genes responsive to DNA-damage and apoptosis

In HPBLs exposed to MCLR for 4 h, the mRNA expression of *p53*, *cdkn1a*, *gadd45a* and *mdm2* was not different from that in the vehicle-treated cells (Table 2). After a 24-h exposure, the expression of *p53* (1.636 ± 0.461), *cdkn1a* (1.523 ± 0.125), *gadd45a* (1.726 ± 0.438) and *mdm2* (1.393 ± 0.076) was up-regulated (Table 2). The mRNA levels of the pro-apoptotic gene *bax* and the anti-apoptotic gene *bcl* were also not changed after a 4-h exposure, while after 24 h the expression of the pro-apoptotic gene *bax* (1.744 ± 0.038) was significantly up-regulated.

3.3.2. Effect of MCLR on the mRNA expression of genes responsive to oxidative stress

No changes in the expression of selected genes responsive to oxidative stress were observed in HPBLs exposed to MCLR for 4 h (Table 2). After a 24-h exposure to MCLR the expression of the genes *cat* (1.576 ± 0.239), *sod1* (1.95 ± 0.054), *gclc* (1.614 ± 0.043), *gsr* (1.585 ± 0.057) and *gpx1* (1.741 ± 0.269) was significantly ($P < 0.05$) higher than in vehicle-treated cells.

4. Discussion

Several previous studies have shown that MCLR induces DNA strand-breaks and oxidative DNA damage *in vivo* and *in vitro* [16,19,35–41]. In the present study we show the induction of DNA strand-breaks and oxidative DNA damage in HPBLs after exposure

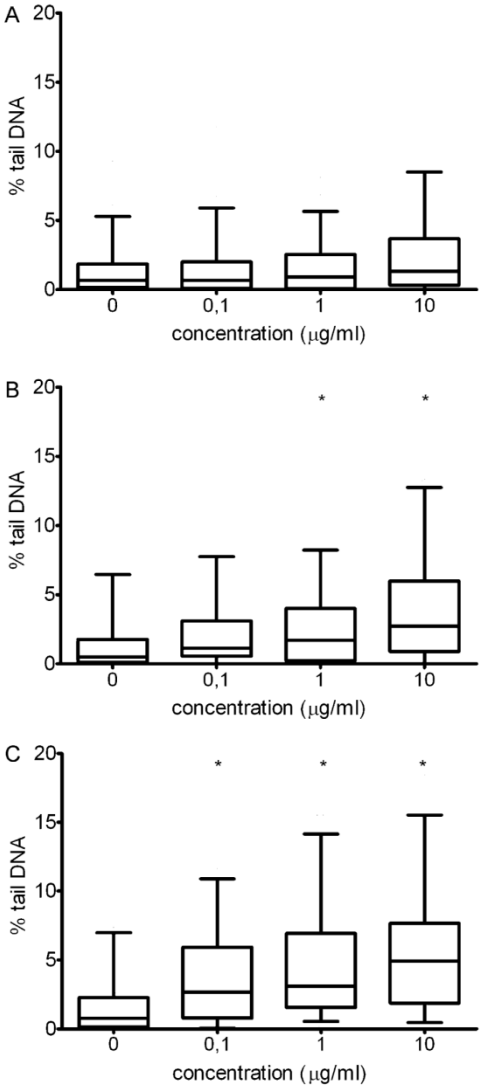

Fig. 1. DNA damage induced by MCLR in HPBLs. Whole blood was exposed to 0, 0.1, 1 and 10 μg/ml MCLR for 4 (A), 6 (B) and 24 h (C). DNA damage is expressed as percent of DNA in tail. Fifty cells were analyzed per experimental point in each of the two independent experiments. Data are presented as quantile box plots. The edges of the box represent the 25th and 75th percentiles, the mean values are represented as a solid line through the box, and the box plot bars represent the 95% confidence intervals. The asterix (*) denotes a significant difference between the vehicle control and MCLR-treated cells (Kruskal–Wallis test, $P < 0.05$). Non-treated control cells did not differ significantly from vehicle control cells (data not shown).

to MCLR. At the molecular level, MCLR induced up-regulation of genes responsive to DNA damage (*p53*, *cdkn1a*, *mdm2*, *gadd45a*) and oxidative stress (*cat*, *sod1*, *gclc*, *gsr*, *gpx1*), and of the gene *bax*, which is associated with apoptosis.

Lankoff et al. [15] reported a correlation between the extent of DNA damage in HPBLs exposed to 25 μg/ml MCLR and the frequency of apoptotic cells, and concluded that the observed DNA damage was related to early apoptosis due to cytotoxicity and not to genotoxicity. However, in our study we observed DNA damage

Table 1
Number of binucleated cells with micronuclei, number of micronuclei (MN), number of nuclear bridges (NPB), number of cells with nuclear buds, and number of nuclear buds per 1000 binucleated peripheral blood lymphocytes after exposure to MCLR (0, 0.1, 1 and 10 μg/ml) for 24 h. Nuclear division index (NDI) was evaluted in 500 cells.

MCLR (24 h)	MNed cells (1000 cells)	MNs (1000 cells)	NPB (1000 cells)	Cells with buds (1000 cells)	Buds (1000 cells)	NDI
0 μg/ml	5.5 ± 2.12	5.5 ± 2.12	2.5 ± 0.71	4.0 ± 1.41	4.0 ± 1.41	2.006 ± 0.00
0.1 μg/ml	6.0 ± 0.00	6.0 ± 0.00	1.5 ± 0.71	8.5 ± 0.71	10.0 ± 1.41	1.975 ± 0.04
1 μg/ml	3.0 ± 1.41	3.0 ± 1.41	2.0 ± 1.41	4.0 ± 0.00	4.5 ± 0.71	1.986 ± 0.00
10 μg/ml	4.5 ± 0.71	4.5 ± 0.71	6.0 ± 2.83	4.0 ± 2.83	4.5 ± 3.54	2.023 ± 0.05
Bleomycin	26 ± 2.83*	28 ± 2.83*	4.0 ± 0.0	13.0 ± 1.41*	13.0 ± 1.41*	1.880 ± 0.06*

* Significant difference between MCLR-treated groups and control (Student's t-test; $P < 0.05$).

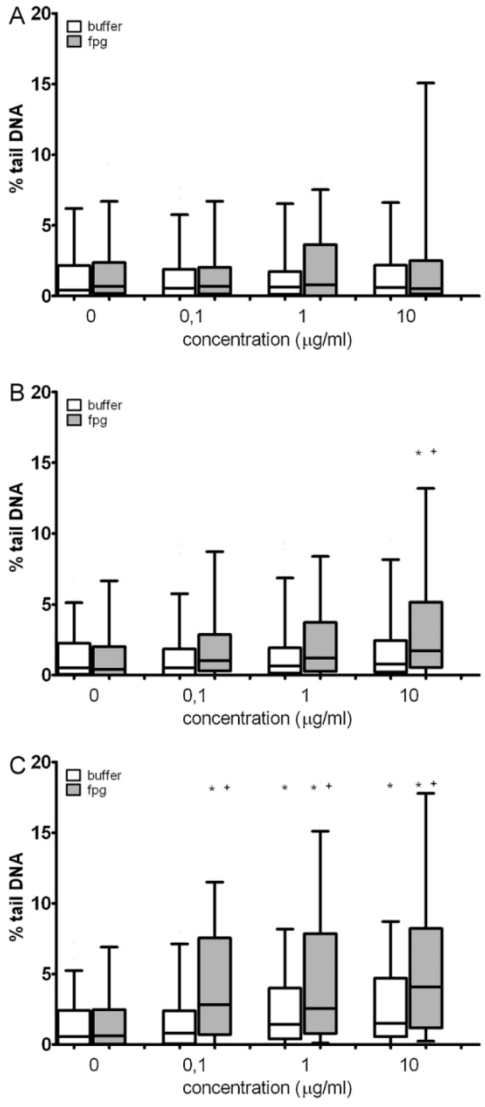

Fig. 2. The level of MCLR-induced DNA strand-breaks with (gray box plots) and without (white box plots) Fpg digestion. Whole blood was exposed to 0, 0.1, 1 and 10 μg/ml MCLR for 4 (A), 6 (B) and 24 h (C), then the modified comet assay was performed as described in Section 2. The levels of DNA strand-breaks and oxidized purines are expressed as percent of DNA in tail. The data are displayed as box plots (for details see the caption of Fig. 1). *Significant difference between MCLR-treated groups and control, +significant difference between Fpg enzyme-digested and non-digested groups (Kruskal–Wallis test, $P < 0.05$).

at much lower concentrations (0.1 μg/ml), at which no cytotoxicity was detected, thus the damage can be ascribed to a genotoxic effect. Similarly, cyanobacterial extracts collected from the Sulejóv water reservoir, Poland, containing MCs (2.5–10 μg/ml) [42] and pure MCLR (1–4 μg/ml) [42,43] induced DNA damage in HPBLs.

The induction of DNA strand-breaks in lymphocytes has been observed also *in vivo*, in mice after a single oral, but not intraperitoneal administration of MCLR [44].

In recent years, the cytokinesis-block micronucleus (CBMN) assay has emerged as a biomarker of chromosome/genome damage relevant to cancer. It is the preferred method for measuring micronuclei (MN), which originate from chromosome fragments or whole chromosomes that lag behind at anaphase during nuclear division [32]. However, new developments indicate that this method can also be used for measuring nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs). NPBs provide a measure of DNA mis-repair, chromosome rearrangement or telomere end-fusion and are assumed to occur when the centromeres of dicentric chromosomes are pulled to opposite poles of the cell at anaphase [34,45]. In the process of nuclear budding, which occurs during S-phase, amplified and/or excess DNA is removed from the nucleus and is a marker of gene amplification [34,45]. In HPBLs no increase

Table 2
Changes in gene expression of *p53*, *mdm2*, *gadd45α*, *cdkn1a*, *bax*, *bcl*, *cat*, *gpx1*, *gclc*, *sod1*, and *gsr* after exposure of HPBLs to MCLR (0 and 1 μg/ml) for 4 and 24 h. The results are expressed as relative mRNA expression normalized to untreated control ±SD.

gene	MCLR conc. (μg/ml)	4 h	24 h
p53	0	1.001 ± 0.061	1.000 ± 0.026
	1	1.037 ± 0.050	1.636 ± 0.461*
mdm2	0	0.960 ± 0.105	1.001 ± 0.049
	1	1.015 ± 0.054	1.393 ± 0.076*
cdkn1a	0	1.002 ± 0.079	1.000 ± 0.020
	1	0.938 ± 0.087	1.529 ± 0.125*
gadd45a	0	1.001 ± 0.042	1.000 ± 0.035
	1	1.004 ± 0.081	1.726 ± 0.438*
bax	0	1.000 ± 0.019	1.003 ± 0.093
	1	0.994 ± 0.077	1.744 ± 0.038*
bcl	0	1.003 ± 0.093	1.000 ± 0.021
	1	0.939 ± 0.097	1.351 ± 0.350
gclc	0	1.000 ± 0.035	1.002 ± 0.071
	1	0.952 ± 0.126	1.588 ± 0.049*
gsr	0	1.001 ± 0.040	1.001 ± 0.052
	1	0.881 ± 0.153	1.585 ± 0.057*
gpx1	0	1.003 ± 0.082	1.002 ± 0.066
	1	1.074 ± 0.089	1.741 ± 0.269*
cat	0	0.944 ± 0.019	1.000 ± 0.018
	1	0.886 ± 0.050	1.576 ± 0.239*
sod1	0	1.003 ± 0.102	1.001 ± 0.038
	1	1.127 ± 0.166	1.950 ± 0.054*

* Significant difference between MCLR-treated groups and control (Student's *t*-test; $P < 0.05$).

in MN, NPBs and NBUDs formation was observed after 24 h of exposure. To our knowledge our study is the first where the effect of pure MCLR on the formation of NPBs and NBUDs has been evaluated.

These results are in agreement with a study in which cyanobacterial extracts containing at least three MCs (MCLR, MCYR and MCRR) also failed to induce MN in isolated HPBLs after a 44-h exposure [46]. Also pure MCLR (1, 10 and 25 μg/ml) did not induce chromosome aberrations in HPBLs [15]. In addition, lack of clastogenic activity has been shown in CHO-K1 cells exposed to pure MCLR and to cyanobacterial extract containing three microcystins (MCLR, MCRR and MCYR) [47,48]. However, the toxins led to an accumulation of abnormal G2/M figures with hypercondensed chromosomes in metaphase-arrested cells, and to a dose-dependent increase in the frequencies of abnormal anaphase, with defective chromosome separation and polyploid cells [47].

In contrast to these studies, treatment of the human lymphoblastoid cell line TK6 with MCLR (5–80 μg/ml) induced an increase in MN frequency and the mutation frequency at the heterozygous thymidine kinase (TK) locus. MCLR specifically induced loss of heterozygosity at the TK locus, but not point mutations or other small structural changes, which indicate that MCLR had a clastogenic effect [49]. The results from two *in vivo* studies are contradictory. Cyanobacterial extracts containing MCs injected i.p. did not increase the frequency of micro-nucleated polychromatic erythrocytes in the peripheral blood in male CBA mice [46], while they significantly increased the frequency of micronuclei in bone-marrow cells of male Kunming mice [35].

Toxicogenomics, the application of gene expression techniques in toxicological studies, facilitates the interpretation of a mode of action of toxic compounds and also allows the prediction of specific toxic effects based on gene-expression changes. The comparison of gene-expression profiles analyses may also provide insight into the mechanisms and pathways altered shortly after exposure [50,51]. Therefore we analyzed the changes in the expression of selected genes involved in the response to DNA damage, oxidative stress and apoptosis, in HPBLs exposed to MCLR (1 μg/ml) for 4 and 24 h. The tumour-suppressor gene *p53* plays a central role in the cellular response to agents or conditions that damage DNA, by activating the transcription of several essential genes controlling cell-cycle arrest/DNA repair and apoptosis. Slight, although significant up-regulation of *p53* gene-expression was observed after 24 h of exposure to MCLR. This is not unusual, as it is known that DNA damage activates the p53 protein predominantly through its phosphorylation by DNA damage-responsive kinases and, to lesser extent, through up-regulation of gene expression [52]. In HepG2 cells, MCLR up-regulated *p53* expression only after short-term exposure (2 h) [53] whereas Chen et al. [54] reported slight up-regulation of *p53* expression in hepatocytes from rats exposed to MCLR for 24 h. In addition to up-regulation of the *p53* gene, it has been reported that sublethal doses of MCLR increased the phosphorylation of the p53 protein in Balb/c mice [55], while Fu et al. [56] detected accumulation of the p53 protein in the rat-hepatocyte BRL-3A cell line exposed to MCLR.

One of the first effects of p53 activation, in nearly all mammalian cell types, is a block in the cell-division cycle. Ellinger-Ziegelbauer et al. [51] reported that in liver of rats exposed to genotoxic and non-genotoxic carcinogens, the p53 down-stream regulated genes, *cdkn1a*, *mdm2* and *gadd45a* were specifically up-regulated by genotoxic carcinogens. The p53 protein directly stimulates the expression of $p21^{WAF1/CIP1}$, an inhibitor of cyclin-dependent kinases (CDKs), which inhibits both G1 to S and G2 to M transitions. $p21^{WAF1/CIP1}$ arrests the cell cycle until DNA damage can be repaired or apoptosis has been initiated [57]. The tumour-suppressor activity of p53 is modulated by *mdm2*, which is a product of a proto-oncogene, amplified or otherwise over-expressed in a significant number of human tumours [58,59]. The *gadd45* gene product interacts with various cell-cycle related proteins and contributes to G2/M cell-cycle arrest, DNA repair and apoptosis [60–62]. The induction of *gadd45a* has been reported to be associated with the oxidative stress-induced pathway [63]. In HPBLs we observed up-regulation of the expression of *cdkn1a*, *mdm2* and *gadd45a* after exposure to MCLR for 24 h, but not after a 4-h exposure. Similar up-regulation of these genes after longer exposure had been previously observed in MCLR-treated HepG2 cells [53], while elevated expression of *cdkn1a* and *gadd45a* has been reported in hepatocytes isolated from MCLR-treated Balb/c mice [54], and in B6.129-*Trp53*$^{+,+}$ N5 mice [64]. These data again add to the evidence of MCLR as a genotoxic agent.

There is evidence that exposure to MCLR can induce apoptosis [36,54,65]. The p53 protein also regulates apoptosis by up-regulating the transcription of pro-apoptotic genes and down-regulating anti-apoptotic genes, including those of the bcl-2 family [66]. In rat hepatocyte BRL-3A cells exposed to MCLR an increased Bax/Bcl-2 protein ratio was observed [56] and in livers of male ICR mice, MCLR treatment resulted in a marked increase in Bax and Bid expression detected by the western blotting [67]. In HepG2 cells, MCLR induced the expression of the *bax* gene, while the *bcl* gene showed a trend towards down-regulation [53]. Elevated expression of the *bax* gene was observed also *in vivo* in hepatocytes of Balb/c mice exposed to MCLR [54]. Similar to these results, we detected a significant increase in the expression of *bax* after a 24-h treatment of HPBL with MCLR, while the expression of the *bcl-2* gene was not significantly changed, compared to the control.

There is strong evidence that MCLR induces ROS formation and oxidative stress; thus it is expected to trigger the cellular antioxidative defence system. It is well documented that exposure to MCLR influences the activities of several antioxidant enzymes such as glutathione peroxidase (GPX) [40,41,68–70], glutathione reductase (GR) [40,69–72], glutathione-S-transferase (GST) [40,41,73], catalase (CAT) [40,41,69–71], superoxide dismutase (SOD) [40,41,69–71], while studies on modulations of gene expression of these enzymes after MCLR exposure are limited. Glutathione, which can exist in either a reduced (GSH) or oxidized (GSSG) form, plays a crucial role in intracellular antioxidant defence systems [74]. The cycling between GSH and GSSG serves to remove free radicals produced due to metabolism of toxic substances, and to protect cells from oxidative injuries [75]. In the presence of GSH, glutathione peroxidases (GPx1–4) catalyze the reduction of organic hydroperoxides or hydrogen peroxide, where upon glutathione disulfide (GSSG) is formed. This can then be reduced to GSH by glutathione reductase (GR) [76]. In the study with female Balb/c mice exposed i.p. to MCLR, elevated hepatic levels of GPX activity and expression were observed [68], while Clark et al. [64] reported a high increase in the expression of two glutathione peroxidase genes, *gpx2* and *gpx3*, after exposure of mice to sublethal doses of MCLR. Our results revealed that in human lymphocytes the expression of both genes, *gpx1* and *gsr*, was up-regulated after 24 h of exposure to MCLR.

The pool of cellular GSH is replenished via two major routes: the above-mentioned reduction of GSSG via GR and via *de novo* synthesis. The synthesis of GSH is regulated by two ATP-dependent enzymes, i.e. glutamate-cysteine ligase (GCLC; also called γ-glutamyl-cysteine synthetase) and glutathione synthase (GS) [77]. Few previous studies provided indirect evidence for the *de novo* GSH biosynthesis after the exposure to MCLR by measuring the expression of GCLC [68,72] or GS enzymes [68], and – corroborating these results – we observed a significant increase in the expression of *gclc* after 24 h of exposure to MCLR also in HPBLs.

When severe oxidative stress occurs, superoxide dismutase (SOD) and catalase (CAT) become more important in the protection of cells than the glutathione redox cycle [78]. To date, there is no report concerning possible changes in the gene expression of

SOD and CAT in cells exposed to MCLR. Our results of gene expression profile of *sod1* and *cat*, after 24 h exposure of HPBLs to MCLR showed significant up-regulation of both enzymes with *sod1* being more pronounced.

In conclusion, exposure of HPBLs to MCLR induced DNA damage that was of oxidative nature, while the formation of micronuclei, nucleoplasmic bridges and nuclear buds was not increased. At the molecular level, exposure of HPBLs to MCLR induced changes in the mRNA expression of p53-regulated DNA damage-responsive genes *cdkn1a*, *gadd45*α and *mdm2*, the apoptosis-associated gene *bax* and genes involved in the antioxidant defence *gpx*, *gsr*, *gclc*, *sod1*, and *cat*. These results provide additional evidence that MCLR should be considered as indirectly genotoxic, via induction of oxidative stress and that lymphocytes are a sensitive target of its adverse effects.

Conflict of interest

The authors report no conflict of interests.

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2.1.4 Cilindrospermopsin sproži odziv pri celicah humanega hepatoma HepG2 na transkripcijskem nivoju

Cylindrospermopsin induced transcriptional responses in human hepatoma HepG2 cells

Alja Štraser, Metka Filipič, Bojana Žegura

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Cianobakterijski toksin cilindrospermopsin (CYN) povzroča genotoksične učinke v številnih testnih sistemih. Preliminarni podatki kažejo, da bi lahko deloval celo karcinogeno, vendar pa je znanje o mehanizmih njegove potencialne karcinogenosti omejeno. Da bi dobili vpogled v mehanizme genotoksičnega in potencialno karcinogenega delovanja CYN, smo analizirali spremembe v izražanju izbranih genov, ki so vključeni v takojšnji-zgodnji odziv in celično signalizacijo, regulacijo celičnega cikla in celične proliferacije, popravljalne mehanizme DNK, apoptozo in celično preživetje ter detoksifikacijske mehanizme. Analiza izražanja genov je bila izvedena po izpostavitvi celic humanega hepatoma HepG2 necitotoksičnim, vendar genotoksičnim koncentracijam CYN (0,5 µg/ml za 12 in 24 h). CYN je povišal izražanje genov takojšnjega-zgodnjega odziva iz genskih družin *FOS* in *JUN*. Spremembe v izražanju tarčnih genov so kazale na sprožitev signalnih poti P53 in NF-κB. Močno povišanje izražanja genov, ki so inducibilni z DNK poškodbami (*GADD45α* in *GADD45β*), inhibitorjev od ciklinov odvisnih kinaz (*CDKN1A* in *CDKN2B*), kinaze kontrolne točke 1 (*CHEK1*) in genov, ki sodelujejo v popravljalnih mehanizmih DNK poškodb (*XPC, ERCC4* in drugi), so nakazovali na ustavitev celičnega cikla, sprožitev popravljalnih mehanizmov DNK (dvoverižnih prelomov in nukleotidno-izrezovalnega popravljanja). Vzorci deregulacije pro- in antiapoptotskih genov niso dali jasnega odgovora, ali CYN povzroča apoptozo. Povišano izražanje metaboličnih genov I. faze (*CYP1A1, CYP1B, ALDH1A2* in *CES2*) in II. faze (*UGT1A6, UGT1A1, NAT1* in *GSTM3*) kaže na njihovo vpletenost v detoksifikacijo in morebitno aktivacijo CYN. Pridobljeni vzorci izražanja genov podajajo nove informacije o celičnem odzivu na izpostavitev CYN.

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Cylindrospermopsin induced transcriptional responses in human hepatoma HepG2 cells

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Detoxification response

ABSTRACT

The newly emerging cyanotoxin cylindrospermopsin (CYN) is showing genotoxic effects in a range of test systems. However, the knowledge on the mechanisms involved is limited. To get insight into the cellular responses to CYN a toxicogenomic analysis of selected genes commonly affected by genotoxic stress was performed on HepG2 cells exposed to a non-cytotoxic but genotoxic concentration of CYN (0.5 μg/ml for 12 and 24 h). CYN increased expression of the immediate-early response genes from the *FOS* and *JUN* gene families and there was strong evidence for the involvement of P53 and NF-κB signaling. Strong up-regulation of the growth arrest and DNA damage inducible genes (*GADD45A* and *GADD45B*), cyclin-dependent kinase inhibitors (*CDKN1A* and *CDKN2B*), checkpoint kinase 1 (*CHEK1*), and genes involved in DNA damage repair (*XPC*, *ERCC4* and others) indicated cell-cycle arrest and induction of nucleotide excision and double strand break repair. Up-regulation of metabolic enzyme genes provided evidence for the involvement of phase I (*CYP1A1*, *CYP1B*, *ALDH1A2* and *CES2*) and phase II (*UGT1A6*, *UGT1A1*, *NAT1* and *GSTM3*) enzymes in the detoxification response and potential activation of CYN. The obtained transcriptional patterns after exposure of HepG2 cells to CYN provide valuable new information on the cellular response to CYN.

1. Introduction

The cyanobacterial toxin cylindrospermopsin (CYN), a potent protein synthesis inhibitor (Froscio et al., 2003; Liang et al., 1999; Runnegar et al., 2002), is increasingly being found in freshwater bodies infested by cyanobacterial blooms worldwide, and was reported to be implicated in human intoxications and animal mortality (Carmichael et al., 2001; Froscio et al., 2001; Hawkins et al., 1985). Evidence for its genotoxic activity and carcinogenic potential is accumulating and was summarized in the review by (Žegura et al. (2011b)). At present, CYN is being included in the revision of the WHO "Guidelines for Drinking-water Quality, chemical hazards in drinking-water" and the US Environmental Protection Agency (EPA) has classified it on the list of compounds with highest priority for hazard characterization (EPA, 2010).

The main target of CYN is the liver, but other organs can also be affected, suggesting it is a general cytotoxin (Falconer et al., 1999; Hawkins et al., 1985; Humpage and Falconer, 2003; Terao et al., 1994). Structurally it is a stable tricyclic alkaloid (415 Da) with several potential sites for reactivity that may form protein and DNA adducts. The later have been identified *in vivo* (Shaw et al., 2000). It contains uracil that could potentially interact with adenine groups in RNA and DNA. This suggests that CYN is a potential genotoxic carcinogen, acting through DNA synthesis interference and induction of mutations (Falconer and Humpage, 2001). CYN was shown to be genotoxic in a range of test systems *in vitro* (Bazin et al., 2010b; Humpage et al., 2000, 2005;Štraser et al., 2011; Žegura et al., 2011a) and *in vivo* (Bazin et al., 2010a; Shaw et al., 2000; Shen et al., 2002) and several studies demonstrated that CYN needs to be activated by cytochrome P450 (CYP450) enzymes to become genotoxic (Bazin et al., 2010b; Humpage et al., 2005; Štraser et al., 2011; Žegura et al., 2011a). Moreover, preliminary data has shown it could have tumor-initiating activity (Falconer and Humpage, 2001) and although there has been an indication of CYN carcinogenicity for humans (Falconer, unpublished data), the studies on CYN carcinogenic activity are scarce and the mechanisms involved are not well understood.

Toxicogenomic approaches represent a promising tool to elucidate mechanisms of toxicity and are becoming increasingly used for hazard identification and risk assessment of genotoxic

Abbreviations: AP-1, activator protein; BER, base excision repair; CDK, cyclin-dependant kinase; CDKI, cyclin-dependant kinase inhibitor; CYN, cylindrospermopsin; CYP450, cytochrome P450; DSB, DNA double-strand break; ER, endoplasmic reticulum; GST, glutathione S-transferase; HPBLs, human peripheral blood lymphocytes; HRR, homologous recombination repair; MMR, mismatch repair; NER, nucleotide excision repair; NHEJ, none-homologous end joining; NF-κB, nuclear factor kappa B; PCNA, proliferating cell nuclear antigen; TGF-B, transforming growth factor B; TNFα, tumor necrosis factor α.

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and carcinogenic properties of different substances (Ellinger-Ziegelbauer et al., 2009; Thybaud et al., 2007). It was shown that using genomic signatures it is also possible to identify carcinogens and identify cellular pathways affected by genotoxic and non-genotoxic carcinogens *in vivo* (Ellinger-Ziegelbauer et al., 2005). The aim of this study was to investigate CYN induced changes in the expression of selected genes from pathways, involved in (i) the immediate-early response and signaling, (ii) regulation of cell-cycle and cell-proliferation, (iii) DNA damage repair apoptosis and cell survival, and (iv) metabolism and detoxification. The gene expression analysis was performed with the metabolically active human hepatoma HepG2 cells, exposed to CYN, using custom quantitative PCR (qPCR) arrays.

2. Materials and methods

2.1. Chemicals

Cylindrospermopsin (CYN) was purchased from Enzo Life Sciences GmbH, Lausen, Switzerland. A 0.5 mg/ml stock solution of CYN was prepared in 50% methanol. William's medium E and glycogen were obtained from Sigma, St. Louis, USA. Penicillin/streptomycin, fetal bovine serum (FBS), L-glutamine and phosphate buffered saline (PBS) were from PAA Laboratories, Dartmouth, USA. Trypsin was from BD-Difco, Le Pont-De-Claix Cedex, France. TRIzol reagent and DNaseI amplification grade were from Invitrogen, Paisley, Scotland, UK. cDNA High Capacity Archive Kit, Power SYBR Green PCR Master Mix and Nuclease-free Water (not DEPC-treated), were from Applied Biosystems, New Jersey, USA. Custom human qPCR-arrays, StellARrays™, were from Lonza, Basel, Switzerland. All other chemical reagents were of the purest grade available. All solutions needed for RNA isolation, DNase digestion, transcription to cDNA and the master mix were prepared in RNase-free water (not DEPC-treated).

2.2. Cell culture and treatment protocol

The metabolically active human hepatoma cell line, HepG2, has retained inducibility and activities of several phase I and phase II xenobiotic metabolising enzymes and it has been shown that several classes of indirect acting genotoxic agents can be detected with this cell line (Knasmüller et al., 2004). HepG2 cells were a gift from Dr. Firouz Darroudi (Leiden University Medical Centre, Department of Toxicogenetics, Leiden, The Netherlands). The cell line was tested for mycoplasma, and was confirmed to be negative. The cells were grown at 37 °C and 5% CO_2 in William's medium E, containing 15% fetal bovine serum, 2 mM L-glutamine and 100 U/ml penicillin/streptomycin. Cells were seeded on T-25 flasks (Corning Costar Corporation, Corning, NY, USA) at a density of 10^6 cells/flask and incubated for 24 h at 37 °C and 5% CO_2. They were washed with 1× PBS, exposed to 0.5 µg/ml CYN and incubated for 12 and 24 h. In each experiment a vehicle control (0.05% methanol) was included. We previously showed that at the exposure conditions selected for this study CYN was not cytotoxic and induced DNA damage (Štraser et al., 2011).

2.3. Total RNA isolation

After the incubation, cells were washed twice with 1× PBS and total RNA was isolated using TRIzol reagent according to the manufacturer's protocol with minor modifications. Glycogen (20 µg/ml) was added to the cell lysate. The RNA was incubated with isopropyl alcohol overnight at −20 °C to precipitate. Purified RNA was stored at −80 °C until analysis.

2.4. DNase digestion and RNA quality and quantity assessment

The isolated RNA was treated with modified DNaseI, amplification grade, according to the manufacturer's protocol, to digest possible contaminating genomic DNA. The concentration and purity of total RNA were measured by spectroscopy using NanoDrop ND-1000 (Thermo Fisher Scientific, Wilmington, USA). RNA integrity was checked with agarose gel electrophoresis (Fig. 1) before gene expression analysis. Total RNA was isolated in 4 parallels for each time point, 3 parallels with purest RNA of highest quality were chosen for further analysis.

2.5. cDNA synthesis

The RNA was transcribed to cDNA using 1.5 µg of total RNA and cDNA High Capacity Archive Kit, according to the manufacturer's protocol. The cDNA synthesis reactions were stored at −20 °C until further analysis.

2.6. Gene selection

A total of 190 genes representing key molecules from selected pathways and functional processes that are known to be activated in response to DNA damage and cell stress, and were expected to be expressed in HepG2 cells, were selected (summarized in supplementary data). The necessary information for selection of the genes was obtained from searching several databases, including NCBI gene, Pubmed and SwissProt, among others. 18S rRNA and *GAPDH* were selected as internal reference genes (normalizer genes). Other normalizer genes were selected from the test genes after the analysis (see below Section 2.8).

2.7. Real-time quantitative PCR (qPCR) reaction

Gene expression was analyzed using custom human 384 well qPCR -arrays (StellARrays™) and qPCR (ABI 7900 HT Sequence Detection System, Applied Biosystems, USA). PCR reaction mixtures were prepared with Power SYBR Green master mix, nuclease-free water (non-DEPC treated) and the template c-DNA. Reagent set-up volumes were calculated according to the manufactures protocol. The conditions for the qPCR were 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s following 60 °C for 1 min, and for the dissociation step 95 °C for 15 s, 60 for 15 s and 95 °C for 15 s.

2.8. Data analysis

For the evaluation of the amplification curves the threshold value for all arrays was set to 0.15. The data was analyzed based on the ΔΔCt method. For both time points 10 normalizer genes (reference genes) were selected using the GPR software, provided by the manufacturer. Statistical significance was calculated by the two tailed Student's *t*-test and the criteria for significance were $p < 0.05$ and absolute ΔΔCt (fold-change) >1.5. Independent experiments were repeated three times.

3. Results and discussion

The objective of this study was to determine which cellular pathways are affected by the cyanobacterial toxin CYN and thereby obtain insight into the mechanisms of CYN genotoxicity and potential carcinogenicity. The investigation was focused on changes in the expression of genes commonly affected by genotoxic stress (genes involved in the immediate-early response, signaling, cell cycle and proliferation, DNA damage repair, apoptosis and cell

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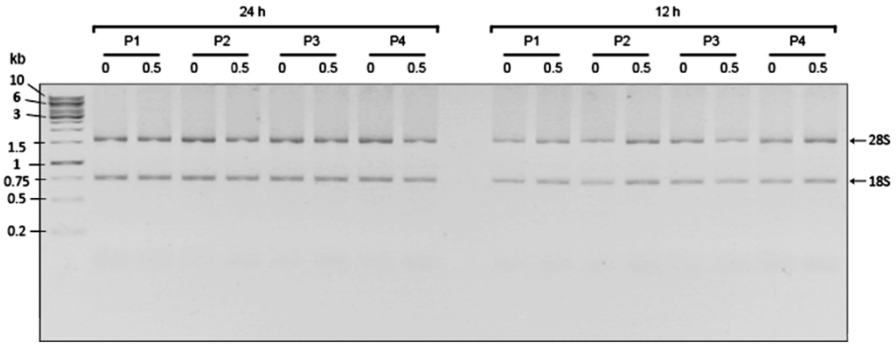

Fig. 1. DNase I digested total RNA (0.5 μg) from all samples (4 parallels from two time points, each parallel contains the vehicle control (0) and the CYN exposed cell sample (0.5)) on 1.5% agarose gel. The two clear bands represent the 28S and 18S rRNA, and show the integrity of the RNA.

survival), and genes involved in the detoxification response to CYN (summarized in the Supplementary data).

More genes were significantly deregulated after 24 h of CYN (0.5 μg/ml) exposure compared to 12 h exposure (Table 1). After 12 h of exposure to CYN, 31% of the selected genes were statistically significant deregulated, but only 17% were deregulated by more than 1.5-fold relative to non-treated control cells (Fig. 2). After 24 h of exposure, 47% of the genes were statistically significant deregulated and 30% were deregulated for more than 1.5-fold (Fig. 2). The expression of 7 genes (signaling and early response: *HGF*, *RAC1*, and metabolism: *XDH, GSTM5, CYP4B1, NNMT* and *FMO3*) was not detected in HepG2 cells under the applied experimental conditions.

The overall gene deregulation patterns were similar at both exposure times, but the deregulation of most of the genes was more pronounced after longer exposure (24 h). This is in line with our previous studies showing more pronounced genotoxic effects of CYN after longer exposure (24 h) (Štraser et al., 2011; Žegura et al., 2011a). The reason could be slow progressive up-take of the toxin by the cells probably through passive diffusion, that was previously reported in Vero cells (Froscio et al., 2009) and primary rat hepatocytes (Chong et al., 2002), and/or the assumingly required metabolic activation of CYN to form DNA reactive metabolites (Bazin et al., 2010b; Humpage et al., 2005; Štraser et al., 2011; Žegura et al., 2011a).

3.1. Early response and signaling pathways

Changes in the expression of genes involved in signaling and immediate-early response were among the strongest deregulations, after both time points of exposure to CYN (Table 1). Transcriptional changes associated with cellular stress are typically regulated by three major transcription factors: the tumor suppressor protein P53; the nuclear factor kappa B (NF-κB); and the activator protein (AP-1) (Welch and Chrylogelos, 2002). AP-1 consists of members of the JUN and FOS protein families, which are encoded by members of the immediate-early response genes. These genes (e.g. *FOS*, *JUN*, *MYC*) are induced rapidly independent of *de novo* protein synthesis (Vogelstein et al., 2000), determining the faith of the cell that can be either cell-cycle arrest, proliferation or apoptosis, or DNA damage repair (Welch and Chrylogelos, 2002). The immediate-early response genes were among the highest up-regulated genes (more than 4-fold) detected after CYN exposure. CYN up-regulated *FOS*, *FOSB* and *JUNB* already after 12 h, and to an even higher degree after 24 h of exposure. In addition, *JUN* was up-regulated after 24 h. The induction of *FOS* is known to be indicative for DNA-damage and is increased by a wide variety of DNA-damaging agents (Hollander and Fornace, 1989), and is showing specificity for genotoxic agents (Dickinson et al., 2004). Also the *JUN* gene was previously reported to be up-regulated by genotoxic agents such as ionizing radiation (Sherman et al., 1990). Increased transcription of *JUN* and *FOS* has been described for two other genotoxic cyanobacterial toxins, the cyclic peptides, microcystin-LR (MCLR) and nodularin (NOD) (for details see (Žegura et al., 2011b)).

CYN also up-regulated the expression of *NFKB1*, the gene encoding for the NF-κB p105 subunit, which is a DNA binding subunit of the NF-κB protein complex, indicating involvement of NF-κB signaling in CYN induced stress response. NF-kB pathways were shown to be activated by DNA damaging agents such as ionizing radiation and DNA topoisomerase I and II inhibitors that cause double strand breaks (DSB), and others such as UV radiation A and B (Habraken and Piette, 2006; Janssens and Tschopp, 2006; Reelfs et al., 2004). Indeed, several genes involved in double strand break repair were induced by CYN, which is discussed in Section 3.3 DNA damage repair.

In line with our previous findings (Štraser et al., 2011) and the findings by Bain et al. (2007), we could not detect any change in the expression of *P53* after exposure to CYN although several P53 target genes were deregulated after CYN exposure (discussed later in detail). The tumor-suppressor protein, P53, plays the central role in the cellular response to damage DNA by activating the transcription of several essential genes controlling cell cycle arrest, DNA repair, senescence, differentiation and apoptosis (Vogelstein et al., 2000). It is known that DNA damage activates the P53 protein predominantly through its phosphorylation and, to lesser extent, through up-regulation of gene expression (Zhou and Elledge, 2000).

In addition, CYN induced up-regulation of three other genes involved in cell signaling *GAB1*, *TGFB2* and *GDF15*. The up-regulation of *GAB-1* was transient. GAB1 is a scaffolding adapter protein that plays a central role in cellular growth response, transformation and apoptosis (Holgado-Madruga and Wong, 2004). Its over-expression was reported to enhance cell growth and promote transformation in NIH3T3 fibroblasts (Holgado-Madruga et al., 1996), to prevent apoptosis in PC12 cells (Holgado-Madruga et al., 1997), and to promote tubulogenesis in epithelial cells (Weidner et al., 1996). *TGFB2* was up-regulated at both time points after CYN exposure with similar fold-increase. *TGFB2* belongs to transforming growth factor Bs (TGF-Bs) that regulate a wide variety of cellular processes (Roberts, 1998), and were shown to inhibit epithelial cell proliferation by delaying or arresting progression through the late portion of G1 (Roberts and Sporn, 1993). *GDF15*, a distant member of the TGF-B superfamily, was up-regulated by

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Table 1
Genes significantly deregulated by CYN exposure for more than 1.5-fold.

Gene symbol	12 h		24 h		Entrez Gene Name
	Fold-change	p-Value	Fold-change	p-Value	
Immediate-early response/signaling					
FOSB	**4.59**	2.34E−02	**8.58**	3.55E−03	FBJ murine osteosarcoma viral oncogene homolog B
FOS	**4.61**	1.70E−02	**4.59**	5.80E−03	FBJ murine osteosarcoma viral oncogene homolog
JUNB	**2.87**	5.95E−03	**4.71**	6.08E−04	Jun B proto-oncogene
TGFB2	**2.27**	9.04E−02	**2.95**	1.66E−03	Transforming growth factor. beta 2
JUN		NS	**2.58**	1.29E−03	Jun proto-oncogene
GDF15		NS	**2.46**	1.64E−03	growth differentiation factor 15
NFKB1		NS	**2.03**	2.31E−03	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
GAB1	**1.84**	4.78E−02		NS	GRB2-associated binding protein 1
Cell cycle/proliferation					
GADD45B	**3.49**	2.01E−02	**4.95**	1.57E−03	Growth arrest and DNA-damage-inducible. beta
GADD45A	**1.68**	9.66E−03	**4.10**	1.52E−04	Growth arrest and DNA-damage-inducible. alpha
CDKN1A		NS	**3.71**	2.28E−03	Cyclin-dependent kinase inhibitor 1A (p21. Cip1)
CDKN2B	**2.27**	1.12E−02		NS	Cyclin-dependent kinase inhibitor 2B (p15. inhibits CDK4)
HUS1	**1.72**	2.13E−02	**2.80**	1.19E−03	HUS1 checkpoint homolog (S. pombe)
CHEK1	<1.5	2.05E−03	**2.42**	6.49E−03	Checkpoint kinase 1
CDK7	<1.5	3.88E−02	**1.94**	1.99E−02	Cyclin-dependent kinase 7
CCNE2	**1.93**	4.66E−04	**1.61**	2.48E−03	Cyclin E2
E2F4		NS	**1.53**	5.80E−03	E2F transcription factor 4. p107/p130-binding
PCNA	>−1.5	3.79E−03	**−1.71**	2.56E−03	Proliferating cell nuclear antigen
CDKN2C		NS	**−1.77**	3.86E−02	Cyclin-dependent kinase inhibitor 2C (p18. inhibits CDK4)
CCNG1		NS	**−1.71**	7.01E−03	Cyclin G1
UHRF1		NS	**−1.71**	2.27E−02	Ubiquitin-like with PHD and ring finger domains 1
TFDP1		NS	**−1.70**	4.66E−07	Transcription factor Dp-1
RAD1		NS	**−1.69**	9.79E−04	RAD1 homolog (S. pombe)
DNA damage repair					
XPC	**3.11**	1.27E−03	**3.08**	6.35E−05	Xeroderma pigmentosum. complementation group C
ERCC4	**2.30**	3.15E−05	**2.96**	6.67E−04	Excision repair cross-complementing rodent repair deficiency. complementation group 4
LIG4	**1.68**	2.00E−02	**1.95**	3.73E−04	Ligase IV. DNA. ATP-dependent
MSH3		NS	**1.51**	1.57E−03	mutS homolog 3 (E. coli)
XRCC2	**3.21**	3.39E−02		NS	X-ray repair complementing defective repair in Chinese hamster cells 2
RAD51	**−2.31**	3.44E−04	**−3.42**	6.23E−05	RAD51 homolog (S. cerevisiae)
MRE11A		NS	**−2.02**	1.08E−04	MRE11 meiotic recombination 11 homolog A (S. cerevisiae)
BRCA2		NS	**−1.66**	4.31E−02	Breast cancer 2. early onset
POLB	**−7.22**	8.31E−03	>−1.5	3.21E−02	Polymerase (DNA directed). beta
Apoptosis/survival					
FAS	**2.73**	3.44E−04	**3.85**	5.45E−05	Fas (TNF receptor superfamily. member 6)
DIABLO	**1.69**	7.71E−04	**2.94**	7.49E−03	Diablo. IAP-binding mitochondrial protein
TIMP1	**1.97**	1.29E−02		NS	TIMP metallopeptidase inhibitor 1
BCL2L1	**1.81**	1.06E−02	**2.43**	1.54E−04	BCL2-like 1
TNF	**51.38**[a]	1.86E−02	**27.11**[a]	1.10E−02	Tumor necrosis factor
TNFAIP3	**2.04**	4.36E−02	**3.41**	3.04E−04	Tumor necrosis factor. alpha-induced protein 3
TNFRSF10A	<1.5	4.04E−03	**1.95**	4.65E−04	Tumor necrosis factor receptor superfamily. member 10a
MCL1		NS	**1.90**	2.04E−04	Myeloid cell leukemia sequence 1 (BCL2-related)
CASP9	**1.54**	5.13E−04	**1.82**	7.28E−05	Caspase 9. apoptosis-related cysteine peptidase
BAK1		NS	**1.81**	3.59E−04	BCL2-antagonist/killer 1
TRADD	<1.5	3.99E−03	**1.78**	6.54E−04	TNFRSF1A-associated via death domain
CASP3		NS	**1.75**	3.58E−02	Caspase 3. apoptosis-related cysteine peptidase
FOXO3	**1.87**	1.35E−02	**1.61**	1.63E−03	Forkhead box O3
CASP8	<1.5	2.98E−02	**1.57**	2.64E−03	Caspase 8. apoptosis-related cysteine peptidase
DDIT3		NS	**1.56**	2.25E−03	DNA-damage-inducible transcript 3
BCL2	**−3.25**	9.99E−03	**−3.12**	8.41E−04	B-cell CLL/lymphoma 2
TNFSF10		NS	**−2.97**	6.16E−04	Tumor necrosis factor (ligand) superfamily. member 10
BID	**−2.25**	3.68E−04	**−2.03**	3.65E−05	BH3 interacting domain death agonist
APAF1	>−1.5	2.94E−03	**−1.73**	1.19E−03	Apoptotic peptidase activating factor 1
CASP7	**−2.54**	2.01E−02		NS	Caspase 7. apoptosis-related cysteine peptidase
Detoxificaton response					
CAT		NS	**4.59**	1.59E−02	Catalase
ALDH1A2	UND		**5.53**	1.36E−02	Aldehyde dehydrogenase 1 family. member A2
CYP1A1	**5.96**	3.66E−04	**2.98**	2.61E−02	Cytochrome P450. family 1. subfamily A. polypeptide 1
CYP1B1	**3.47**	3.27E−03		NS	Cytochrome P450. family 1. subfamily B. polypeptide 1
UGT1A6	**6.81**	9.63E−03		NS	UDP glucuronosyltransferase 1 family. polypeptide A6
TXNRD1	<1.5	1.96E−02	**2.09**	1.02E−03	Thioredoxin reductase 1
NAT1	<1.5	4.84E−03	**1.94**	4.84E−04	N-acetyltransferase 1 (arylamine N-acetyltransferase)
GCLC	<1.5	2.21E−03	**1.93**	2.44E−03	Glutamate-cysteine ligase. catalytic subunit
CES2	<1.5	1.58E−02	**1.77**	1.84E−02	Carboxylesterase 2
GSTM3	**1.52**	1.67E−02	**1.69**	8.59E−05	Glutathione S-transferase mu 3 (brain)
UGT1A1	**1.67**	3.28E−02		NS	UDP glucuronosyltransferase 1 family. polypeptide A1
CYP2A6	**−3.74**	1.17E−02		NS	Cytochrome P450. family 2. subfamily A. polypeptide 6
CYP2A13		NS	**−3.72**	4.00E−02	Cytochrome P450. family 2. subfamily A. polypeptide 6
CYP3A43		NS	**−2.48**	1.52E−02	Cytochrome P450. family 3. subfamily A. polypeptide 43

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Table 1 (*continued*)

Gene symbol	12 h		24 h		Entrez Gene Name
	Fold-change	p-Value	Fold-change	p-Value	
CYP3A7		NS	**−2.36**	4.74E−04	Cytochrome P450. family 3. subfamily A. polypeptide 7
GSTM2	>−1.5	5.18E−03	**−2.16**	2.78E−04	Glutathione S-transferase mu 2 (muscle)
CYP2F1	**−2.43**	1.10E−03	**−2.03**	3.41E−03	Cytochrome P450. family 2. subfamily F. polypeptide 1
GSTA2		NS	**−1.76**	4.24E−03	Glutathione S-transferase alpha 2
CES1	>−1.5	2.08E−02	**−1.74**	3.95E−03	Carboxylesterase 1
GNMT		NS	**−1.63**	3.76E−02	Glycine N-methyltransferase
SULT1A1		NS	**−1.58**	1.10E−03	Sulfotransferase family. cytosolic. 1A. phenol-preferring. member 1

[a] The gene was undetectable (UND) in the control population and was assigned a Ct number of 40 to determine the fold-change.

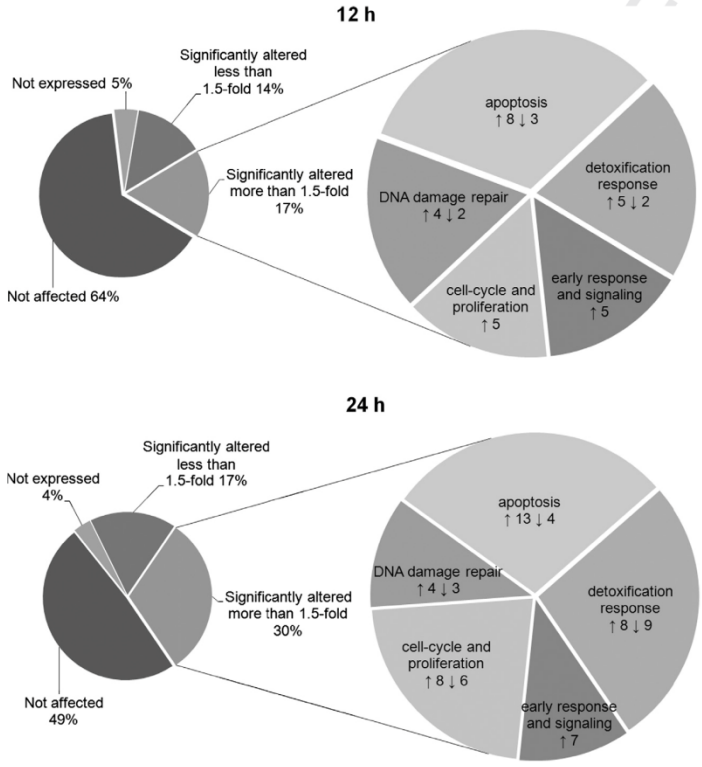

Fig. 2. Schematic representation of the transcriptional response of HepG2 cells to CYN exposure.

CYN after 24 h exposure. This gene is a transcriptional target gene of P53 and was described to be induced by genotoxic stress (Osada et al., 2007; Yang et al., 2006).

3.2. Cell cycle and proliferation

Only a few published studies report CYN influence on the cell-cycle and proliferation. Lankoff et al. (2007) described that CYN significantly decreased the mitotic index and proliferation in CHO-K1 cells. Also in our previous studies exposure to CYN (0.5 μg/ml) for 24 h significantly decreased the nuclear division index in HepG2 cells (Štraser et al., 2011) and at even lower concentrations (0.1 μg/ml) in human peripheral lymphocytes (HPBLs) (Žegura et al., 2011a).

After 12 h of exposure to CYN five genes involved in cell-cycle regulation were significantly up-regulated (Table 1). After 24 g exposure additional four genes were up-regulated and six were down-regulated (Table 1). One of the up-regulated genes was *CHEK1*, the protein product of which is a checkpoint kinase that is activated in response to DNA damage and can thereafter modulate the activity of a number of proteins including P53, providing a link between ATR sensing of DNA damage and P53 checkpoint activity (Ranuncolo et al., 2008). Activation of the sensor kinases ATM and ATR (ataxia-telangiectasia and rad3-related), and the checkpoint kinases CHEK1 and CHEK2 prevents G1/S or G2/M transitions (Malumbres and Barbacid, 2009). However, *ATM* and *CHEK2* transcription was not affected by CYN.

The main target of P53 upon DNA damage is *CDKN1A*, that encodes $P21^{WAF1/CIP1}$, an important cyclin dependant kinase (CDK) inhibitor (CDKI), that inhibits the cell-cycle at the G1/S and the G2/M transitions (Michael and Oren, 2002; Vogelstein et al., 2000). CYN up-regulated *CDKN1A* after 24 h, which is in line

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with previous reports (Bain et al., 2007; Štraser et al., 2011; Žegura et al., 2011a). Here we show that another CDKI, *CDKN2B*, was up-regulated after 12 h, while CYN down regulated *CDKN2C* after 24 h. These two CDKIs encode the proteins $P15^{INK4B}$ and $P18^{INK4C}$, respectively, which are able to bind and inhibit two G1-phase CDKs, namely CDK4 and CDK6 (Serrano et al., 1993), preventing cells from transition from G1 to S phase.

Exposure to CYN also induced strong time-dependent up-regulation of the growth arrest and DNA-damage inducible genes *GADD45A* and *GADD45B*. Up-regulation of *GADD45A* after exposure to CYN was previously reported in HepG2 cells (Štraser et al., 2011), HPBLs (Žegura et al., 2011a) and human dermal fibroblasts (Bain et al., 2007). It is known that *GADD45* genes (*GADD45A*, *GADD45B* and *GADD45G*) are rapidly induced by a wide spectrum of genotoxic agents (Dickinson et al., 2004; Ellinger-Ziegelbauer et al., 2005; Heise et al., 2012) and play pivotal role in negative growth control, either dependent or independent of P53 (Liebermann and Hoffman, 1998; Zhan et al., 1999). The protein products of *GADD45A* and *GADD45B*, together with GADD45G, cooperate in activation of S and G2/M cell cycle checkpoints following exposure of cells to genotoxic stress (Vairapandi et al., 2002). Their up-regulation and the up-regulation of *CHEK1*, *CDKN1A* and *CDKN2B* after CYN exposure strongly indicates activation of cell-cycle checkpoints and cell-cycle arrest.

CYN down-regulated the cyclin G1, *CCNG1*. *CCNG1* is a known transcriptional target of P53 (Gordon et al., 2000) and it was reported that P53 can suppress *CCNG1* through microRNA (miRNAs) induction (Gramantieri et al., 2007). The down-regulation of *CCNG1* in the present study indicates growth inhibition by CYN, as suppression of cyclin G1 in cancer cell lines was previously shown to result in the reduction of proliferation, and in apoptosis induction (Gordon et al., 2000). On the other hand, the expression of cyclin E2 (*CCNE2*) and the CDK 7 (*CDK7*) was up-regulated by CYN. E-type cyclin availability is limited to the early stages of DNA synthesis, when they bind and activate CDK2 (Harbour et al., 1999), which is thought to be essential for the G1/S phase transition (Hochegger et al., 2008). CDK7 binds to cyclin H and was shown to be required for the activation of CDK2 and CDK1, and is involved in the S/G2 and G2/M phase transitions (Larochelle et al., 2007).

CYN exposure also increased the gene expression of the transcription factor *E2F4* and decreased the expression of the transcription factor Dp-1 (*TFDP1*). These two genes belong to the E2F family of transcription factors, that play a pivotal role in regulating the expression of genes involved in the G1/S transition and DNA synthesis (Cam and Dynlacht, 2003). As E2F4 is a major transcriptional repressor, inhibiting cell-cycle progression, DNA replication, mitosis, stimulating cell-cycle exit and differentiation (Cam and Dynlacht, 2003), its up-regulation indicates inhibition of cell-cycle progression by CYN.

In addition, CYN decreased the expression of *UHRF1*, the gene encoding Ubiquitin-like with PHD and ring finger domains 1 (UHRF1), which has a critical role in cell cycle progression as it promotes G1/S transition (Arita et al., 2008). As knockdown of *UHRF1* was shown to suppress cancer cell proliferation (Unoki et al., 2009), its down-regulation by CYN again indicates CYN mediated growth inhibition. The *UHRF1* gene is a direct target of E2F1 and was shown to be down-regulated by P53 through $P21^{WAF1/CIP1}$ (Unoki et al., 2009), whose mRNA transcription was up-regulated by CYN, as already discussed. UHFR1 physically interacts with the proliferating cell nuclear antigen (PCNA) (Sharif et al., 2007) at the DNA replication fork or DNA repair sites where it recognizes hemi-methylated CpG (Unoki et al., 2009). Also *PCNA* was down-regulated after CYN exposure. PCNA interacts with several cell cycle proteins and was originally characterized as a DNA sliding clamp, an essential component of the eukaryotic DNA replisome (Kelman and O'Donnell, 1995). Subsequently however, it was shown to be involved in many processes including DNA repair and cell-cycle control (Maga and HÃĽbscher, 2003).

CYN also deregulated components of the 9-1-1 cell-cycle checkpoint response complex, which resembles the PCNA sliding clamp, further coupling cell-cycle arrest with DNA-damage repair. *HUS1* was up- and *RAD1* down-regulated and the third component *RAD9A* was slightly down-regulated, however less than 1.5-fold. In response to DNA damage the 9-1-1 complex is loaded onto DNA at sites of damage, where it facilitates the activation of CHEK1 and thereby coordinates checkpoint activation and DNA repair (Parrilla-Castellar et al., 2004).

3.3. DNA damage repair

The main mechanisms of DNA repair are the base excision repair (BER), nucleotide excision repair (NER), direct damage reversal, DNA double-strand break (DSB) repair and mismatch repair (MMR) (Christmann et al., 2003).

BER is responsible for removing DNA-damaged bases. In our study *POLB* that is involved in BER pathways (Podlutsky et al., 2001), was significantly down-regulated after 12 h of CYN exposure. None of the other selected genes involved in BER were significantly deregulated, suggesting a minor role of BER in CYN induced DNA damage repair.

From the selected genes involved in NER, *XPC* (global genome nucleotide excision repair) and *ERCC4* were significantly up-regulated. NER functions to repair bulky DNA-adducts and intra-strand cross-links (Christmann et al., 2003). *XPC* up-regulation was the highest among the DNA-repair associated genes at both time points. It is a P53 target and is inducible by UV light, ionizing radiation, alkylating agents, and benzo[a]pyrene diol peroxide (Amundson et al., 2002; Wang et al., 2003). Its protein product is known to be involved in the recognition of bulky lesions (Amundson et al., 2002). This supports the hypothesis, that CYN forms DNA adducts, which is indicated by its structure (Falconer and Humpage, 2001; Humpage et al., 2000). The formation of DNA adducts after CYN exposure was shown *in vivo* in DNA extracted from mice liver after i.p. exposure to CYN (Shaw et al., 2000). NER requires the breakage and rejoining of DNA strands via structure-specific DNA repair endonucleases such as XPF that is encoded by the gene *ERCC4*. Also the expression of *ERCC4* was increased after CYN exposure at both time points. The induction of NER after CYN induced DNA-adducts would introduce transient single strand breaks that could give positive results seen in the comet assay in several studies (Bazin et al., 2010a,b; Humpage et al., 2005; Shen et al., 2002; Štraser et al., 2011; Žegura et al., 2011a).

Most of the DNA-damage repair associated genes deregulated by CYN were however involved in DSB repair mechanisms. DSBs are the most detrimental form of DNA damage as they can lead to chromosomal breakage and rearrangement (Dasika et al., 1999). Double-strand breaks are expected to be induced by CYN as it was shown to cause micronuclei formation *in vivo* and *in vitro* (Bazin et al., 2010a; Humpage et al., 2005; Shen et al., 2002; Štraser et al., 2011; Žegura et al., 2011a). Induction of DSBs initiates fine tuned networks that lead to repair by homologous recombination (HRR) or non-homologous end joining (NHEJ), checkpoint activation and cell-cycle arrest, apoptosis mostly via P53, activation of mitogen activated protein kinase, and the transcription factors AP-1 and NF-kB (Habraken and Piette, 2006; Norbury and Hickson, 2001), whose involvement in the cellular response to CYN was indicated in this study. CYN exposure up-regulated the gene (*LIG4*) coding for ligase IV that is involved in NHEJ, in which it together with XRCC4 forms a complex that relegates DSBs. The gene coding for XRCC4 (*XRCC4*) was not affected by CYN at the tested conditions. Most of the DSBs, generated by genotoxic agents, cannot be directly relegated and are processed by the

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MRE11-Rad50-NBS1 complex (Maser et al., 1997), which also functions in initiating HRR. One of the components of the complex, *MRE11A*, was down-regulated by CYN after 24 h of exposure. Also the gene *RAD51* was down-regulated by CYN, while a paralog of *RAD51*, *XRCC2*, was up-regulated. The human RAD51 protein forms nucleofilaments, binds to the DNA, promotes interaction with a homologous region on an undamaged DNA molecule and catalyzes strand-exchange events (Christmann et al., 2003).

MMR is a postreplication DNA repair mechanism responsible for the removal of base mismatches caused by spontaneous or induced base damage and replication errors, chemically induced DNA lesions, cross-links, UV-induced photoproducts, purine adducts, 8-oxoguanine and other base modifications (for details see (Christmann et al., 2003)). One component of the MMR system, the mutS homolog 3 (*MSH3*), was up-regulated after 24 h of CYN exposure. The product of this gene is a component of the DNA-lesion recognition complex MutSβ that typically binds to longer insertion/deletion mismatches (Palombo et al., 1996). It is not clear whether MMR is inducible by genotoxic stress and up-regulation of *MSH3* following exposure to genotoxic agents has not been reported before.

3.4. Apoptosis and cell survival

The gene deregulations of pro- and anti-apoptotic genes do not allow for clear interpretation whether CYN induces apoptosis or not (Table 1). Exposure to CYN clearly induced transcriptional changes of genes involved in apoptosis and survival, several of them known targets of the transcription factors P53 and NF-κB. In contrast to P53 activation, which is in terms of cell death and survival associated with the induction of apoptosis, is NF-κB generally considered to have anti-apoptotic activity (Pahl, 1999). Being simultaneously activated these two signaling pathways with generally opposing outcomes must be highly regulated and it was shown that P53 and NF-κB inhibit each other's ability to stimulate gene expression, which is controlled by relative levels of the transcription factors or by regulation at the transcriptional level (Webster and Perkins, 1999).

Both transcription factors are induced by the tumor necrosis factor α (TNFα), a potent proinflammatory cytokine that plays an important role in immunity and inflammation, and in the control of cell proliferation, differentiation and the extrinsic apoptotic pathway (Klefstrom et al., 1997). The gene encoding TNFα, *TNF*, showed the strongest up-regulation of all CYN deregulated genes. However, it should be noted that in the control cells *TNF* was expressed at the limit of detection for which a CT value of 40 was assigned, to calculate the fold-change.

Other genes from the extrinsic apoptotic pathway were also deregulated by CYN. *TRADD*, the expression of which leads to NF-κB activation, and its overexpression to apoptotic cell death (Van Antwerp et al., 1998), and *CASP8* involved in the activation of the cascade of executive caspases were up-regulated after 24 h of exposure to CYN. Also *FAS* and *TNFRSF10A*, encoding the TNFR superfamily members 6 and 10a, were up-regulated following CYN exposure. These results indicate the triggering of the extrinsic apoptotic pathway by CYN. On the other hand, CYN exposure increased transcription of the anti-apoptotic gene *TNFAIP3*, a gene whose expression is rapidly induced by TNFα, and decreased expression of the pro-apoptotic gene *TNFSF10* that encodes the cytokine TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) that binds to several receptors to induce apoptosis (Kimberley and Screaton, 2004). TRAIL is a direct target of the transcription factor FOXO3 (previously known as FKHRL1), whose gene (*FOXO3*) was up-regulated by CYN. This transcription factor promotes apoptosis trough induction of either death-receptor molecules such as FAS and TRAIL or key regulators of the intrinsic pathway (Brunet et al., 1999; Dijkers et al., 2000; Modur et al., 2002).

The intrinsic pathway is strictly controlled by the BCL-2 family proteins that can also modulate the extrinsic pathway through BID. CYN exposure significantly deregulated 5 of the 6 selected genes encoding proteins from the BCL-2 family. The toxin down-regulated the anti-apoptotic *BCL2* and up-regulated two other anti-apoptotic members *BCL2L1* (encoding BCL-X(L)) and *MCL1*. The pro-apoptotic *BID* was down-regulated and *BAK1* up-regulated, while BAX *was* up-regulated by CYN for less than 1.5-fold. The lack of *BAX* induction is consistent with the study by Bain et al. (2007), who showed that CYN significantly increased *BAX* expression in HepG2 cells and in human dermal fibroblasts only at 10-fold higher (5 μg/ml) concentrations then used in the current study. On the other hand, CYN significantly up-regulated both genes, *BAX* and *BCL-2*, in HPBLs with the expression of *BCL-2* being more pronounced (Žegura et al., 2011a). The ratio of death antagonists (BCL-2, BCL-XL, MCL-1 and A-1) to agonists (BAX, BAK, BAD, BID and BCL-XS) determines whether a cell will respond to an apoptotic signal (Kroemer, 1997), however, the deregulations induced by CYN in the current study did not show clear prevalence of either signals.

The signaling pathways critical for cell survival are mediated in part by the composition and integrity of the extracellular matrix and the action of its components on specific cell adhesion receptors. Consistently, the matrix metalloproteinases (MMPs), a group of peptidases involved in degradation of the extracellular matrix, or their inhibitors (TIMPs), have been suggested to regulate apoptosis. The gene for TIMP1 (Tissue inhibitor of matrix metalloproteinase-1), *TIMP1*, was up-regulated at the earlier time point of CYN exposure. Transcription of this gene is highly inducible in response to many cytokines and hormones and was also shown to be up-regulated upon exposure to several genotoxic carcinogens (Ellinger-Ziegelbauer et al., 2004, 2005). In contrast to its well-established anti-tumor effect, TIMP-1 is also able to promote cell proliferation of a wide range of cell types (Hayakawa et al., 1992), and has an anti-apoptotic function (Guedez et al., 1998; Jiang et al., 2002). *TIMP1* over-expression induced up-regulation of *BCL2L1* in B cells (Guedez et al., 1998), which is consistent with our results.

All pathways to apoptosis converge on the activation of caspases (Klefstrom et al., 1997; Youle and Strasser, 2008). All caspase genes included in our study were deregulated by CYN. Following the intrinsic pathway progression, cytochrome C release from the mitochondria leads to the activation of the apoptotic protease-activating factor-1 (APAF1) into an apoptosome that activates caspase-9, which than leads to the activation of down-stream initiator and effector caspases (Youle and Strasser, 2008). While *APAF1* was down-regulated after 24 h CYN exposure, *CASP9*, encoding caspase-9, was up-regulated at both time points. The extrinsic pathway can bypass the mitochondrial step and activate caspase-8 directly. In addition to *CASP9* and *CASP8* CYN up-regulated *CASP3* after 24 h of exposure, while *CASP7* was down-regulated after 12 h. Furthermore, CYN exposure increased the expression of *DIABLO* at both time points. Its protein product (direct IAP binding protein with low pI), also referred to as SMAC: second mitochondria-derived activator of caspases) is released from the mitochondria along with cytochrome C, and promotes caspase activation (Du et al., 2000; Verhagen et al., 2000).

After longer exposure (24 h) CYN increased the expression of the DNA damage inducible transcript 3 *DDIT3* (also known as *GADD153* or *CHOP*), which encodes a leucine zipper transcription factor (McCullough et al., 2001). *DDIT3* was originally identified based on its induction following treatment of cells with growth arresting and DNA damaging agents (Luethy and Holbrook, 1992), however the gene is most responsive to endoplasmic retic-

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ulum (ER) stress as it is inducible by agents that directly (Chen et al., 1992; Halleck et al., 1997; Price and Calderwood, 1992) or indirectly (Bruhat et al., 1997; Carlson et al., 1993) lead to impairment in the protein folding in the ER. As CYN is a protein synthesis inhibitor that probably impairs the translational step (Froscio et al., 2001, 2003; Runnegar et al., 2002), it may cause ER stress. Coupling ER stress to apoptosis, elevated *DDIT3* expression was shown to result in the down-regulation of *BCL2* expression (McCullough et al., 2001) that was also shown after CYN exposure in this study; however here we show that *BCL2* was down-regulated already before the up-regulation of *DDIT3*. *DDIT3* up-regulation was also reported to be concomitant with depletion of cellular glutathione, and exaggerated production of reactive oxygen species, however the evidence suggests that this was due to reduced transcription of the *BCL2* gene (McCullough et al., 2001). This suggests that possible CYN induced ER stress, caused by its protein synthesis inhibition activity, would be the cause of the glutathione depletion, reported before (Humpage et al., 2005; Runnegar et al., 1995).

3.5. Detoxification response and metabolism

Literature data show increasing evidence that CYN toxic and genotoxic activity is depending on CYP450-metabolism but it is not clear which isoforms are involved. Different broad-spectrum CYP450 inhibitors showed protective effects against the toxicity (Froscio et al., 2003; Norris et al., 2002; Runnegar et al., 1995) and genotoxicity (Bazin et al., 2010b; Humpage et al., 2005) of CYN. In addition, no genotoxic effects were observed in systems, with low metabolic enzyme activity (Bazin et al., 2010b; Fessard and Bernard, 2003; Lankoff et al., 2007).

The selected CYP450 isoforms included in this study are mostly highly inducible enzymes and are known as activators of numerous xenobiotic compounds and play an important role in the oxidative metabolism of numerous xenobiotics. Exposure to CYN induced deregulation of several isoforms (Table 1); supporting previous assumptions that CYP450s are involved in CYN metabolic. In previous studies CYN was shown to up-regulate the expression of *CYP1A1* and *CYP1A2* in HepG2 cells (Štraser et al., 2011) and HPBLs (Žegura et al., 2011a). In contrast to the previous studies, in the present study the up-regulation of *CYP1A1* was more pronounced after 12 h of exposure than after 24 h and we could not confirm the up-regulation of *CYP1A2*. The discrepancies are probably due to the low expression of *CYP1A2* that was also detected in the previous studies, and different methods and chemistries used for the detection of changes in gene expression. In addition, up-regulation of *CYP1B1* after 12 h of exposure was observed, providing evidence for the involvement of this isoform in addition to *CYP1A1* in the metabolic activation of CYN. Interestingly, *CYP1A1* and *CYP1B1* were also the two isoforms most strongly up-regulated by the pro-carcinogen benzo[a]pyrene (BaP) in HepG2 cells (Song et al., 2012).

The mRNA expression of several analyzed CYP450 isoforms was significantly down-regulation by CYN; *CYP2A6* after 12 h, *CYP2F1* after both time points, and *CYP2A13*, *CYP3A43* and *CYP3A7* after 24 h. The mechanisms involved in CYP450 suppression are poorly understood. The expression of CYP450 genes can be down-regulated after exposure to a variety of chemicals (Riddick et al., 2003), as a pathophysiological response to stress signals, adaptive homeostatic response to oxidative stress or as part of a tightly regulated physiological pathway (Morgan, 2001). It is not clear which stimuli regulate which isoforms of CYP450. A decrease in the expression of hepatic CYP450s has been found in acute phase infection and inflammation with the involvement of NF-κB, TNF-α and other inflammatory cytokines but the underlying mechanism has yet to be elucidated (Ke et al., 2001; Morgan, 2001; Riddick et al., 2004). As we observed significant up-regulation of the genes for NF-κB and TNF-α (*NFKB* and *TNF*) this could correlate with the down-regulation of the CYP450s.

At the earlier time point phase II enzymes from the UDP-glucuronosyl-transferase family (*UGT1A6* and *UGT1A1*), and glutathione S-transferase mu 3 (*GSTM3*) were significantly up-regulated. The up-regulation of *UGT1A6* and *UGT1A1* was transient in contrast to *GSTM3* that was up-regulated also after 24 h. In general, conjugation with phase II enzymes is considered the detoxification phase of xenobiotic metabolism, although in certain situations, it could result in activated metabolites and increase in toxicity (Hinson and Forkert, 1995; Rushmore and Tony Kong, 2002; Schilter et al., 1993). It is not known if CYN is also activated by phase II enzymes or if its toxicity or genotoxicity are diminished by their activity, but our results provide evidence for their involvement in the metabolic response to CYN exposure. Likely other phase I and II enzymes are involved in the CYN detoxification response as after longer exposure (24 h) two other phase I enzyme genes, the gene coding for aldehyde dehydrogenase 1A2 (*ALDH1A2*), and carboxylesterase 2, *CES2*, and the phase II enzyme N-acetyltransferase 1 *NAT1* were up-regulated, while there was a decrease in the expression of the metabolic enzyme genes *GSTM2*, *GSTA2*, *CES1*, *GNMT* and *SULT1A1*.

Apart from *GSTM3* that was up-regulated and *GSTM2* and *GSTA2* that were down-regulated, none of the other glutathione S-transferase (GST) genes was significantly affected by CYN (Table 1). The GSTs participate in a detoxification pathway that acts via the conjugation of the substance with glutathione. One mechanism of CYN toxicity was suggested to be the inhibition of glutathione synthesis (Humpage et al., 2005; Runnegar et al., 1995), which can lead to increased oxidative stress and consequently genotoxicity. The observed up-regulation of the catalase gene (*CAT*), a primary antioxidant enzyme, indicates the induction of oxidative stress (Table 1). In addition, thioredoxin reductase, *TXNRD1*, a key enzyme in oxidative stress control that is required for effective DNA binding of redox-sensitive transcription factors, including p53 and NF-κB (Arner and Holmgren, 2006; Hayashi et al., 1993; Ueno et al., 1999), was also up-regulated. Up-regulation of the gene coding for glutamat–cysteine ligase (*GCLC*), the first rate limiting enzyme in glutathione synthesis, indicates possible response of the cells to a depletion of glutathione and an increase of its biosynthesis, after longer exposure to CYN. Decreased glutathione content in the cells can result from either increased oxidation of glutathione, increased efflux, formation of glutathione-conjugates or decreased synthesis. There is only limited data about the induction of oxidative stress by CYN. Humpage et al. (2005), showed that there was no increase in lipid peroxidation, an indicator for oxidative stress, in primary mouse hepatocytes after CYN exposure. In HPBLs CYN induced significant increase in the expression of genes *GCLC*, *GSR*, *GPX1* and *SOD1* after 24 h of exposure, while the mRNA level of *CAT* was not changed (Žegura et al., 2011a). Because of the lack of induction of the other genes involved in oxidative stress response (*NOS2* and *SOD1*) and the glutathione detoxification and antioxidant pathway (*GSTs*, *GSS*, *GSR*, *GPXs* and *GGT1*) in our study the results suggest that CYN induces only minor oxidative stress; however the mechanisms involved need to be further elucidated.

4. Conclusion

The transcriptional responses to low doses (0.5 μg/ml) of CYN provide new insights in the mechanisms of CYN toxicity. CYN exposure induced the immediate-early response (genes from the *FOS* and *JUN* families) and there is strong evidence for the involvement of P53 and NF-κB signaling as several down-stream regulated target genes of these transcription factors were found deregulated. Although different cellular stress factors can result

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in the activation of these transcriptional factors, the induction of several genes associated with DNA-damage repair indicate response to DNA-damage and genotoxic stress and subsequent cell-cycle arrest and induction of NER and DSB repair. This is supported by the strong up-regulation of growth arrest and DNA damage inducible genes (*GADD45A* and *GADD45B*), CDKIs (*CDKN1A* and *CDKN2B*) and *CHEK1*, and genes involved in DNA damage repair (*XPC* and *ERCC4*). The deregulation patterns of pro- and anti-apoptotic genes did not give a clear answer. The genes involved in the processes of apoptosis were not severely affected by CYN. Several up-regulated genes indicate the triggering of the extrinsic (*FAS*, *TNF*, *FOXO3*, *TRADD* and *TNFRSF10A*) and intrinsic apoptotic pathway (*APAF1*, *CASP9* and *DIABLO*), while the deregulation pattern of the *BCL2* family genes and several others is directed in favor of apoptosis suppression. The results, regarding detoxification response, provide evidence for the involvement of phase I (*CYP1A1*, *CYP1B*, *ALDH1A2* and *CES2*) and phase II (*UGT1A6*, *UGT1A1*, *NAT1* and *GSTM3*) enzymes in the activation and detoxification of CYN. After longer exposure (24 h) CYN could induce possible depletion of glutathione and minor oxidative stress that may be the consequence of ER stress response, indicated by the up-regulation of *DDIT3*.

Our results provide valuable new information on the cellular response to CYN exposure. However, to get more precise insight in the mechanisms of CYN toxicity future studies should be focused on the expressions of proteins and enzyme activities, in particular taking into account that CYN is a potent protein synthesis inhibitor, which can have strong impact on final toxic effects of this toxin.

5. Declaration of interest

The authors report no conflict of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tiv.2013.05.012.

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2.1.5 Vpliv cilindrospermopsina na povzročanje oksidativnih DNK poškodb in apoptoze pri celicah HepG2

The influence of cylindrospermopsin on oxidative DNA damage and apoptosis induction in HepG2 cells

Alja Štraser, Metka Filipič, Irena Gorenc, Bojana Žegura

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Cianobakterijski citotoksin in močan zaviralec proteinske sinteze, cilindrospermopsin (CYN), se vse pogosteje pojavlja v površinskih vodah po vsem svetu. Zaradi njegove genotoksične aktivnosti in potencialne karcinogenosti, predstavlja potencialno nevarnost za ljudi. Mehanizmi njegove genotoksične aktivnosti še vedno niso dobro poznani. Da bi ugotovili, ali CYN povzroča poškodbe DNK preko sprožitve oksidativnega stresa, smo merili nastanek reaktivnih kisikovih zvrsti (ROS) s sondo DCFH-DA in nastanek oksidiranih purinov z modificiranim testom komet z encimom Fpg pri celicah HepG2. CYN je statistično značilno povišal nastajanje ROS, količina pa je postopno naraščala s časom izpostavitve. Kljub temu v tem času (4 h) nismo zaznali povišane ravni oksidiranih purinov. Po 12 in 24 h izpostavitvi je CYN povzročil povišano raven poškodb DNK ne glede na encimsko razgradnjo s Fpg, kar kaže na to, da oksidativni stres nima znatne vloge pri nastanku poškodb DNK. Poleg tega smo analizirali, ali CYN povzroči apoptozo. Toksin je statistično značilno povišal mitohondrijski membranski potencial (MMP) po 12 in 24 h izpostavitve, medtem ko sprememb v aktivnosti kaspaz 3 in 7 ni povzročil. Prav tako nismo opazili sprememb v številu apoptotskih celic, ki smo jih določali z barvanjem z Annexin V in PI. Rezultati nakazujejo, da poškodbe DNK, ki jih povzroča CYN, niso oksidativne narave. Ugotovitev, da toksin ne povzroča apoptoze pri genotoksičnih koncentracijah, potencira tveganje za ljudi in živali še posebej pri dolgodobni izpostavitvi nizkim koncentracijam.

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The influence of cylindrospermopsin on oxidative DNA damage and apoptosis induction in HepG2 cells

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HIGHLIGHTS

- Cylindrospermopsin enhanced ROS formation in HepG2 cells.
- The DNA damage induced by cylindrospermopsin was not of oxidative nature.
- Cylindrospermopsin did not induce apoptosis at genotoxic concentrations.

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ABSTRACT

Cylindrospermopsin (CYN) a potent cyanobacterial cytotoxin and protein synthesis inhibitor is increasingly being found in surface freshwaters worldwide. Due to its genotoxic activity and potential carcinogenicity it was recognized as a potential threat to humans. However, the mechanisms of CYN genotoxicity are not well understood. We explored whether CYN at non-cytotoxic exposure conditions causes DNA damage through induction of oxidative stress and whether it induces apoptosis in HepG2 cells. With the DCFH-DA probe a significant increase in the intracellular formation of reactive oxygen species (ROS) was observed, which steadily increased with incubation. Induction of oxidative DNA damage was determined with the modified comet assay with formamidopyrimidine glycosylase (Fpg) digestion. No DNA damage was observed after 4 h exposure to CYN. After 12 and 24 h exposure, CYN (at 0.25 and 0.5 μg mL^{-1}) induced significant increase of DNA strand breaks, but not oxidative DNA damage, suggesting minor role of oxidative stress in CYN mediated genotoxicity. CYN also significantly increased the mitochondrial membrane potential (MMP), determined with the JC-1 probe, while no induction of caspase 3 and 7 activity and no increase in the number of apoptotic cells, measured with Annexin V/PI staining, could be determined. These results show that at non-cytotoxic concentrations CYN induced DNA damage was not the consequence of oxidative stress and that CYN did not induce apoptosis, which may add to the hazard of this toxin, as cells with damaged DNA are not removed from the population, enhancing the risk of mutations and consequently carcinogenesis.

1. Introduction

The newly emerging cyanobacterial toxin cylindrospermopsin (CYN) is increasingly being recognized as a potential threat to drinking water safety (Žegura et al., 2011b). CYN was first identified as the probable cause of a severe case of human poisoning in Australia in 1979 (Krohn et al., 1999), and was since then found to be implicated in several cases of human intoxications and animal mortality (Hawkins et al., 1985; Carmichael et al., 2001; Griffiths and Saker, 2003). It is produced by several freshwater cyanobacterial species and is nowadays more and more often found also in temperate regions of the world due to climate change and increasing eutrophication of water bodies. The main target organ of CYN was long thought to be the liver, but it seems to be a general cytotoxin as other organs such as the kidney, lung, thymus, spleen, adrenal glands, intestinal tract, the immune system and the heart can also be affected (Hawkins et al., 1985; Terao et al., 1994; Falconer et al., 1999; Humpage et al., 2000).

Abbreviations: CYN, cylindrospermopsin; BER, base excision repair; CYP450, cytochrome P450; DCF, 2′,7′-dichlorofluorescein; DCFH-DA, 2′,7′-dichlorfluorescein-diacetate; DSB, double strand break; Fpg, formamidopyrimidine glycosylase; HPBLs, human peripheral blood lymphocytes; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide; LDH, lactate dehydrogenase; MHP, mitochondrial hyperpolarisation; MMP, mitochondrial membrane potential; NER, nucleotide excision repair; PI, propidium iodide; ROS, reactive oxygen species; STS, staurosporine; t-BOOH, tert-butyl hydroperoxide.

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This 415 Da tricyclic alkaloid is a potent protein synthesis inhibitor (Runnegar et al., 2002; Froscio et al., 2003), and has been shown to be genotoxic *in vitro* (Humpage et al., 2000; 2005; Bazin et al., 2010; Štraser et al., 2011; Žegura et al., 2011a) and *in vivo* (Shaw et al., 2000; Shen et al., 2002). Its genotoxic activity has generally been shown only in metabolically competent test systems, which implies that it is pro-genotoxic (Humpage et al., 2005; Bazin et al., 2010; Štraser et al., 2011; Žegura et al., 2011a). In addition, preliminary evidence (Falconer and Humpage, 2001) suggests that the toxin is potentially carcinogenic. However the mechanisms involved in CYN genotoxic and especially carcinogenic activity are still poorly understood and need to be elucidated. The World Health Organization (WHO) included CYN in the revision of the WHO "Guidelines for Drinking-water Quality, chemical hazards in drinking-water", but there is still insufficient information for the classification of CYN as a carcinogen by the International Agency for Research on Cancer (IARC). Due to its adverse effects the U.S. Environmental Protection Agency (EPA) classified CYN on the list of compounds with highest priority for hazard characterization (EPA, 2010). All these facts indicate that information on the mechanisms of its genotoxic and potential carcinogenic activity is urgently needed.

CYN contains several potential sites for reactivity that may form DNA and protein adducts (Shaw et al., 2000; Falconer and Humpage, 2001) and can therefore act as a direct DNA reactive genotoxin and/or by secondary mechanisms such as induction of intracellular ROS formation and disturbance of DNA repair fidelity, cell cycle control or apoptosis. In this respect the present study focuses on the induction of oxidative stress by CYN and its involvement in the CYN induced DNA damage in the metabolically competent human hepatoma cell line, HepG2. As reactive oxygen species (ROS) and the resulting oxidative stress play a pivotal role in apoptosis, which is triggered by specific DNA lesions and is a prominent route for removal of damaged cells upon excess DNA damage (Roos and Kaina, 2006), we also aimed to reveal the influence of CYN on the induction of apoptosis.

2. Materials and methods

2.1. Chemicals

Cylindrospermopsin (CYN) was from Enzo Life Sciences GmbH, Lausen, Switzerland. A 0.5 mg mL^{-1} stock solution of CYN was prepared in 50% methanol. William's medium E, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), ethylenediaminetetraacetic acid (EDTA), dimethylsulfoxide (DMSO), 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide (JC-1), tert-butyl hydroperoxide (t-BOOH) and staurosporine (STS) were obtained from Sigma, St. Louis, USA. Penicillin/streptomycin, foetal bovine serum (FBS), L-glutamine and phosphate buffered saline (PBS) were from PAA Laboratories, Dartmouth, USA. Trypsin was from BD-Difco, Le Pont-De-Claix Cedex, France, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was from GIBCO®, Life Technologies, Carlsbad, California, USA. Triton X-100 was from Fisher Sciences, New Jersey, USA. Annexin V-FITC and propidium iodide (PI) were from BD Biosciences Pharmingen™, San Diego, CA, USA. Apo-ONE® Homogeneous Caspase-3/7 Assay and Ethidium-bromide solution were from Promega, Madison, Wisconsin, USA. Normal melting point agarose (NMP), low melting point agarose (LMP) and 2′,7′-dichlorfluorescein-diacetate (DCFH-DA) were from Invitrogen, Paisley, Scotland. Formamidopyrimidine glycosylase (Fpg) was a gift from Dr. Andrew R. Collins (Department of Nutrition, Faculty of Medicine, University of Oslo, Norway) and concentrations of the enzyme were prepared according to his protocol. All other chemical reagents were of the purest grade available and all solutions were made using Mill-Q water.

2.2. Cell culture and treatment

HepG2 cells were a gift from Dr. Firouz Darroudi (Leiden University Medical Centre, Department of Toxicogenetics, Leiden, The Netherlands). The cells were grown at 37 °C and 5% CO_2 in William's medium E containing 15% foetal bovine serum, 2 mM L-glutamine and 100 U mL^{-1} penicillin/streptomycin. Before each experiment, cells were seeded on appropriate cell culture plates at specified densities, which is described below, separately for each method. After the overnight incubation at 37 °C in 5% CO_2, the growth medium was replaced with fresh medium containing 0, 0.125, 0.25 and 0.5 μg mL^{-1} CYN and incubated for additional 12 and 24 h. In each experiment, a vehicle control (growth medium containing 0.05% methanol) and a positive control (STS (1 μM and 0.5 μM for 12 h and 24 h, respectively) for the apoptosis and cell death experiments and t-BOOH (0.1 mM for 30 min) for the modified comet assay) was included. The exposure conditions were the same for all experiments except for the determination of ROS formation, which is described below, and the additional time point of 4 h was included in the comet assay.

2.3. Intracellular ROS formation

The formation of intracellular ROS was measured using a fluorescent probe, DCFH-DA as described by Osseni et al. (1999) with minor modifications (Žegura et al., 2004). In brief, HepG2 cells were seeded at a density of 1500 cells/well into black 96-well plates (Nunc, Naperville IL, USA). After 24 h of incubation at 37 °C in 5% CO_2, cells were incubated with 20 μM DCFH-DA in PBS. After 30 min, DCFH-DA was removed and cells were treated with CYN (0.05, 0.1 and 0.5 μg mL^{-1}) in PBS. A vehicle control (0.05% methanol) and a positive control t-BOOH (0.5 mM) were included in each experiment. For kinetic analyses the plates were maintained at 37 °C and the fluorescence intensity was determined every 30 min using a microplate reading spectrofluorimeter (Tecan, Genios) at the excitation wavelength of 485 nm and the emission wavelength of 530 nm. Independent experiments were preformed in 5 replicates and were repeated three times.

2.4. Comet assay

HepG2 cells were seeded at a density of 60 000 cells/well into 12-well microtiter plates (Corning Costar Corporation, Corning, NY, USA). At the end of the exposure the level of oxidative DNA damage was determined with the modified comet assay as described by Collins et al. (1993) with minor modifications (Žegura et al., 2003). In brief, the cells were harvested and embedded in LMP on slides, lysed and after the lysis the slides were washed three times for 5 min with endonuclease buffer (40 mM HEPES-KOH, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg mL^{-1} bovine serum albumin, pH 8.0). Then 50 μl of formamidopyrimidine glycosylase (Fpg) was added, covered with a cover glass and incubated for 30 min, at 37 °C. In parallel all the slides were exposed to enzyme buffer as a control. The slides were then processed as described earlier (Žegura et al., 2003). Three independent experiments were performed for each of the treatment conditions. The DNA damage was expressed as % of tail DNA. For direct comparison of Fpg digested and undigested samples, normalization of the mean and median values of the % tail DNA of samples to corresponding vehicle controls was performed. There were no differences between mean and median values; therefore only the results of mean values are shown.

2.5. Cell death analysis by Annexin V staining

HepG2 cells were seeded at a density of 400 000 cells/well into 6-well plates (Corning Costar Corporation, Corning, NY, USA). At the end of the exposure floating and adherent cells were collected by trypsinization and resuspended in binding buffer (0.1 M Hepes (pH 7.4), 1.4 M NaCl, and 25 mM CaCl2) at approximately 10^6 cells mL^{-1}. Into 100 µl of the cell suspension 5 µl of Annexin V and 10 µl of PI were added, mixed and incubated for 15 min. After incubation 200 µl of binding buffer was added and the externalization of phosphatidyl serine was analyzed by flow-cytometry. The positive control was separately stained with only Annexin V (channel FL-1), only PI (channel FL-2), and both, for the compensation settings of the two signals. Flow cytometric analysis was carried out on a FACSCalibur flow cytometer (BD Biosciences Pharmingen™, San Diego, CA, USA). In each sample 10^4 events were recorded and analysis was performed using CellQuest Pro software (BD Biosciences). Dot-plots (FL-1H versus FL-2H) were generated and using quartile statistics the cells were divided into live cells (lower left corner), early apoptotic cells (lower right corner), late apoptotic cells (upper right corner) and necrotic cells (upper left corner). Cell death was determined as the percentage of cells stained positive for either Annexin V or PI or both. Three independent experiments were performed for each of the treatment conditions.

2.6. JC-1 assay for mitochondrial membrane potential analysis

The cells were seeded on black 96-well plates (Nunc, Naperville IL, USA) at the density of 25 000 cells/well. After the incubation the culture medium was discarded and JC-1 probe was employed according to the manufacturer's instruction. Mitochondrial membrane potential (MMP) was monitored by determining the relative amounts of dual emissions from mitochondrial JC-1 monomers (green fluorescence, excitation 485 nm and emission 535 nm) or aggregates (red fluorescence, excitation 535 nm and emission 595 nm) using a fluorescence spectrophotometer (Tecan, Genios). Mitochondrial depolarization was defined as an average decrease in the red to green fluorescence intensity ratio. Independent experiments were performed in five replicates and were repeated three times.

2.7. Caspase 3/7 activity

Caspase 3/7 activity was determined using Apo-ONE® Homogeneous Caspase-3/7 Assay. Cells were seeded on black 384 well plates (Corning Costar Corporation, Corning, NY, USA) at the density of 3000 cells/well in four replicates. After the exposure the assay was performed according to the manufacturers' protocol. After adding the reagent to the wells the plate was incubated in the dark for 3 h. Fluorescence was measured at 485 nm excitation and 530 nm emission, gain 50, using a microplate reading spectrofluorimeter (Tecan, Genios). In parallel the MTT assay was performed as previously described (Štraser et al., 2011), to determine differences in cell viability between control and CYN exposed groups. The fluorescence (485/530 nm) was normalized to the determined cell number. Independent experiments were performed in four replicates and were repeated two times.

2.8. Statistical analysis

Statistical significance between treated groups and the control was determined by One-way analysis of variance and Dunnett's Multiple Comparison Test, using GraphPad Prism 5 (GraphPad Software).

3. Results

3.1. Induction of reactive oxygen species (ROS)

Induction of intracellular formation of ROS by exposure to CYN was explored by the fluorescent probe DCFH-DA. The probe is hydrolyzed by intracellular esterases to a non-fluorescent product DCFH, which is in the presence of ROS rapidly oxidized to the highly fluorescent 2′,7′-dichlorofluorescein (DCF). The DCF fluorescence intensity is proportional to the amount of ROS formed intracellularly. H_2O_2 is the principle ROS responsible for the oxidation of DCFH to DCF (LeBel et al., 1992). A dose dependent statistically significant increase of DCF fluorescence intensity was observed in cells treated with 0.05, 0.1 and 0.5 µg mL^{-1} CYN already after 30 min of exposure (Fig. 1), which steadily increased with incubation time. After 5 h incubation, the fluorescence intensity at the highest dose of CYN was about five times higher than in the control cells. The positive control (0.5 mM t-BOOH) induced about 11-fold increase in DCF fluorescence over the control cells.

3.2. Induction of oxidative DNA damage

Induction of oxidative DNA damage was studied with the modified comet assay with the purified DNA damage specific enzyme, Fpg, which recognizes and excises oxidized purines (Collins et al., 1996). Digestion with Fpg significantly increased the level of DNA strand breaks in the control and in treated cells and these additional DNA strand breaks correspond to oxidized purines. After 4 h of exposure, none of the applied CYN concentrations induced increase in the amount of DNA strand breaks (without enzyme digestion) and oxidative DNA damage (Fig. 2). After 12 and 24 h of exposure a significant increase in DNA strand breaks and oxidative DNA damage was detected at the highest two concentrations of CYN (Fig. 2A). However, when we normalized the mean values of % tail DNA to the corresponding vehicle controls, there was no significant difference between undigested and Fpg-digested samples (Fig. 2B). This shows that the DNA damage caused by CYN was unlikely to be due to oxidative stress.

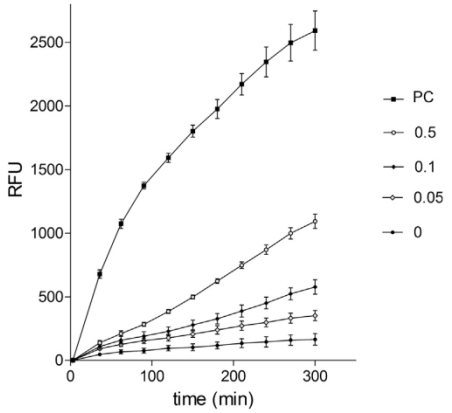

Fig. 1. CYN-induced increase of DCF fluorescence in HepG2. The HepG2 cells were pretreated with DCFH-DA (20 µM) for 30 min, washed and then exposed to different concentrations of CYN (0.05, 0.1 and 0.5 µg mL^{-1}). CYN concentrations that induced significant increase in DCF-fluorescence compared to the vehicle control (0) are shown. Tert-butyl hydroperoxide (0.5 mM t-BOOH) was used as the positive control (PC). DCF fluorescence intensity was measured at 30 min intervals during the 5 h incubation.

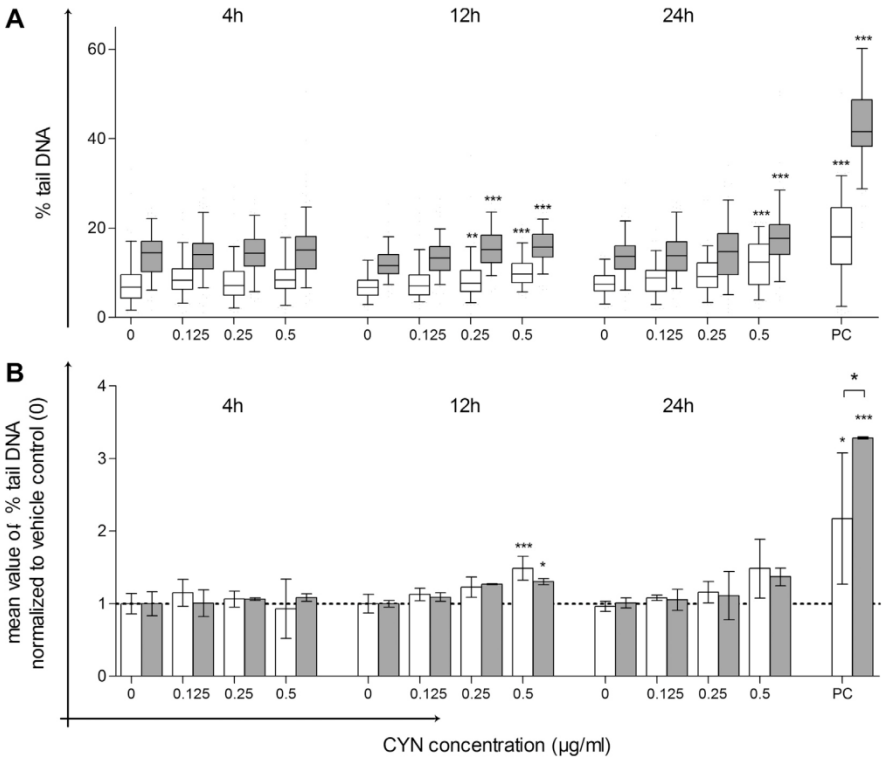

Fig. 2. (A) The level of CYN-induced DNA strand breaks without (white box plots) and with formamidopyrimidine glycosylase (Fpg) digestion (gray box plots). The cells were exposed to CYN (0.125, 0.25 and 0.5 µg mL^{-1}) for 4, 12 and 24 h, then the modified comet assay was performed. Tert-butyl hydroperoxide (0.5 mM) was used as the positive control (PC). The levels of DNA strand breaks and oxidized purines are expressed as percent of tail DNA (% tail DNA). Fifty cells were analyzed per experimental point in each of the three independent experiments. Data are presented as quantile box plots. The edges of the box represent the 25th and 75th percentiles, the median is a solid line through the box, and the error bars represent 95% confidence intervals. (B) Mean values of the % tail DNA were normalized to the corresponding vehicle controls (0) to equalize the basal levels of DNA strand breaks and to enable direct comparison of Fpg digested and undigested samples. Significant difference (1-way ANOVA; Dunnett's Multiple Comparison test) between CYN-treated cells and the vehicle control (0), and between Fpg digested and undigested samples is indicated by $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

3.3. Induction of cell death

Annexin V/PI staining and flow cytometric analysis was used to determine CYN induced phosphatidylserine externalization, which is an early biomarker of apoptosis induction, and increased membrane permeability, which indicates cell death. There was no difference in total cell death in CYN exposed and control cells (Fig. 3A). There were no significant changes in the number of early or late apoptotic or necrotic cells after exposure to CYN (Fig. 3B). The apoptotic agent STS induced significant increase in early and late apoptotic cells and in total cell death.

The loss of mitochondrial membrane potential (MMP) was measured using the fluorescent lipophilic cation dye JC-1. JC-1 exists in two forms with different emission spectra: monomeric with green-fluorescence at low membrane potential, and in "J-aggregates" with red-fluorescence at higher potentials. Apoptotic cells emit mainly green fluorescence, while live cells emit both red and green fluorescence. CYN significantly affected the mitochondrial membrane potential (MMP) after both exposure times (Fig. 4A). However, instead of MMP loss it induced elevation of MMP or mitochondrial membrane hyperpolarisation (MHP) at concentrations 0.25 and 0.5 µg mL^{-1} after 12 h, and 0.5 µg mL^{-1} after 24 h of exposure. The positive control STS induced significant loss of MMP (Fig. 4A).

The activity of the effector caspases 3 and 7 was measured with the ApoOne assay. At the applied exposure conditions CYN induced no significant changes in the caspase 3 and 7 activities (Fig. 4B), indicating no induction of apoptosis, while STS induced pronounced increase in the caspase 3 and 7 activities.

4. Discussion

Several recent studies indicated that the toxicity of CYN may be associated also with the induction of oxidative stress (Gutiérrez-Praena et al., 2012a,b). It is well known that oxidative stress is associated with the induction of genotoxic effects as well as with the interference with apoptotic processes that are both implicated in carcinogenesis. In this study we aimed to reveal whether CYN induced DNA damage is mediated by induction of oxidative stress and how CYN influences the apoptotic processes.

Our study showed that CYN induced dose and time dependent intracellular ROS formation that was detected already after 30 min of exposure. Gutiérrez-Praena et al. (2012a,b) described increased ROS formation in HUVEC (Gutiérrez-Praena et al., 2012a) and CaCo2 (Gutiérrez-Praena et al., 2012b) cells at 0.375 µg mL^{-1} and 1.25 µg mL^{-1} of CYN, respectively, however in their studies ROS formation was determined after 24 h exposure. The

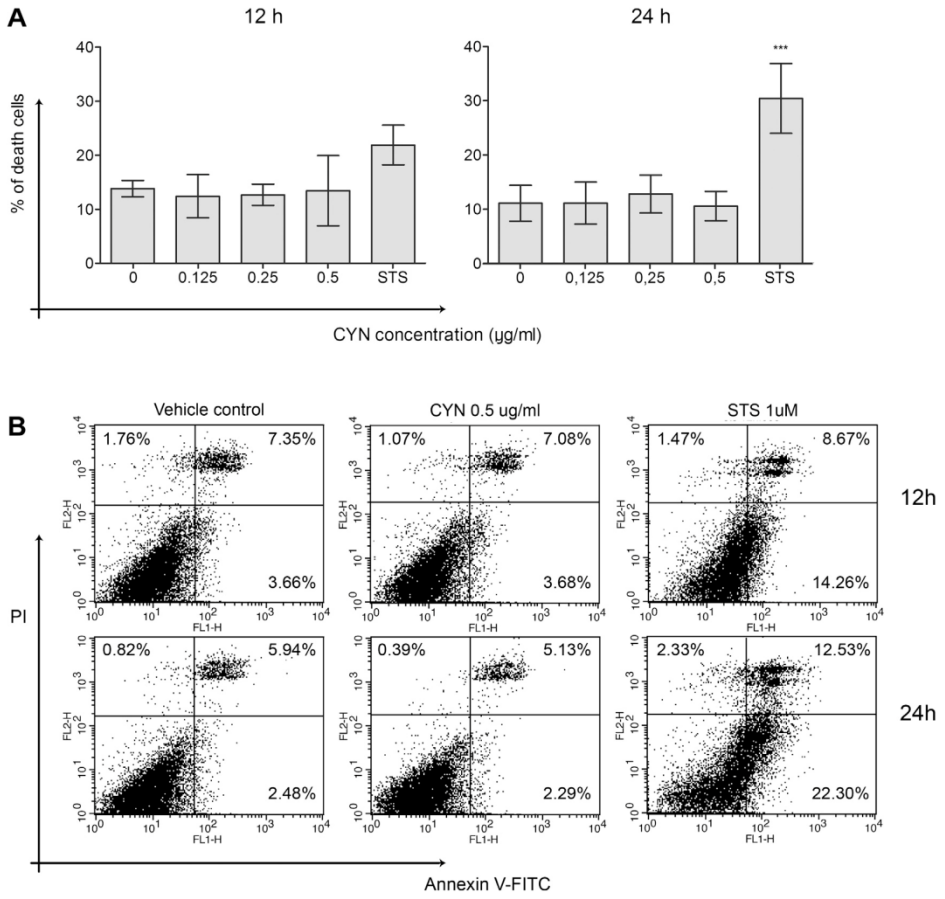

Fig. 3. Annexin V/PI staining. The HepG2 cells were exposed to CYN (0.125, 0.25 and 0.5 µg mL^{-1}) for 12 and 24 h, harvested and stained with Annexin V-FITC/PI. Staurosporine (1 µM/12 h and 0.5 µM/24 h) was used as the positive control. (A) % of death cells was determined as the percentage of cells stained positive for either Annexin V or PI or both. Significant difference (1-way ANOVA; Dunnett's Multiple Comparison test) between the positive control (PC) and the vehicle control (0) is indicated by ***$P < 0.001$. (B) Representative dot-plots (FL-1H versus FL-2H) obtained after 12 and 24 h of cell exposure to the vehicle control (VC), CYN (0.5 µg mL^{-1}) and the positive control (PC) are shown. The four quartiles represent live cells (lower left corner), early apoptotic cells (lower right corner), late apoptotic cells (upper right corner) and necrotic cells (upper left corner).

mechanism of CYN mediated ROS induction is not clear. It is known that CYP450 enzymes reactions are a significant source of ROS formation (Parke, 1994; Olinski et al., 2002; Klaunig and Kamendulis, 2004). As CYP450 enzymes are considered to play a role in the metabolic activation of CYN (Humpage et al., 2005; Bazin et al., 2010; Štraser et al., 2011; Žegura et al., 2011a), these reactions may be a source of ROS formation after CYN exposure. It has also been shown that CYN inhibited glutathione synthesis in primary rat and mouse hepatocytes (Runnegar et al., 1995; Humpage et al., 2005), which can also lead to increased oxidative stress. However, the inhibition of glutathione synthesis by CYN is controversial as in HUVEC (Gutiérrez-Praena et al., 2012a) and CaCo-2 cells (Gutiérrez-Praena et al., 2012b) glutathione content and γ-glutamylcysteine synthetase activity were reported to be increased by CYN exposure.

To explore whether the increased amount of ROS can induce oxidative DNA damage the modified comet assay was employed, using the purified oxidative DNA damage specific enzyme Fpg, which catalyzes the excision of oxidized purines (Collins et al., 1993). Although clear increase in ROS production was observed during the 5 h exposure, after 4 h exposure no DNA strand breaks or oxidative DNA damage were observed. The results are in line with other published data that show CYN to induce toxic and genotoxic effects after at least 12 h of exposure (Humpage et al., 2000; 2005; Bazin et al., 2010; Štraser et al., 2011; Žegura et al., 2011a). The reason might be slow progressive up-take of the toxin by the cells, probably through passive diffusion (Chong et al., 2002; Froscio et al., 2009), and/or the assumingly required metabolic activation of CYN (Humpage et al., 2005; Bazin et al., 2010; Štraser et al., 2011; Žegura et al., 2011a).

After 12 and 24 h exposure to CYN a significant increase in DNA damage was observed in Fpg digested and non-digested DNA. However, the increase in the DNA damage as well as oxidized purines over the control levels were comparable, suggesting that there is no considerable oxidative DNA damage induced by CYN. This result is supported also by our recent toxicogenomic study in which we found that exposure to CYN induced up-regulation of only two of the selected genes involved in the oxidative stress response, catalase (*CAT*) and thioredoxin reductase (*TXNRD1*), while no alterations in transcriptional responses of the many other selected

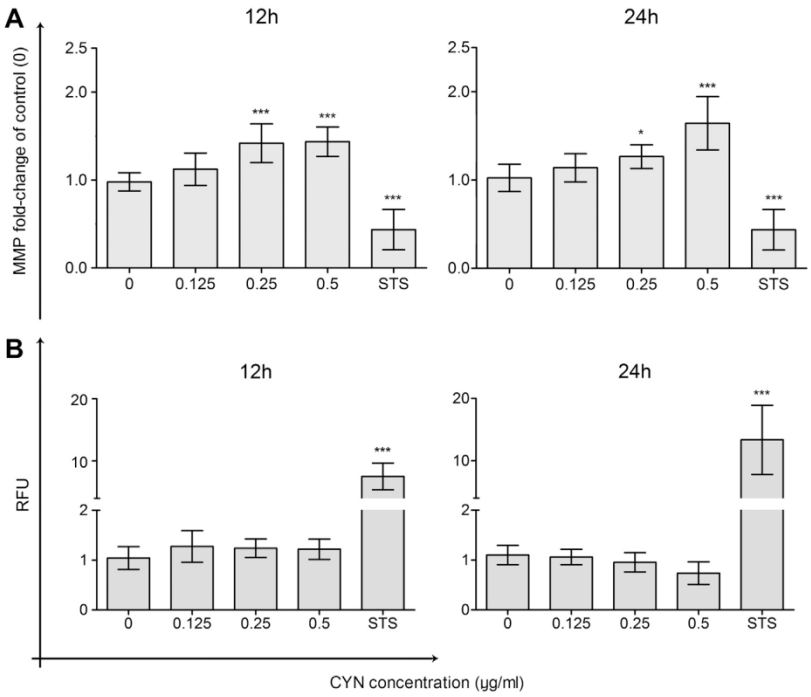

Fig. 4. CYN influence on cell death. The HepG2 cells were exposed to CYN (0.125, 0.25 and 0.5 µg mL^{-1}) for 12 and 24 h. Staurosporine (1 µM/12 h and 0.5 µM/24 h) was used as the positive control. (A) Changes in the mitochondrial membrane potential (MMP) were measured spectroflurometricaly, using the JC-1 dye. MMP fold-change of the vehicle control (0) is shown. (B) Caspase 3/7 activity was measured using the ApoOne assay. Significant difference (1-way ANOVA; Dunnett's Multiple Comparison test) between CYN-treated cells and the vehicle control (0) is indicated by $^{*}P < 0.05$ and $^{***}P < 0.001$.

genes involved in the oxidative stress response, glutathione detoxification and antioxidant pathways were detected (Štraser et al., submitted for publication). Also Humpage et al. (2005) reported no induction of oxidative cellular damage by CYN as no lipid peroxidation was observed in primary mouse hepatocytes.

Oxidative stress induces apoptotic cell death in various cell types (for review see (Slater et al., 1995; Dröge, 2002)). Only three studies reported CYN induced apoptosis in Chinese hamster ovary cells, CHO-K1 (Fessard and Bernard, 2003; Lankoff et al., 2007; Gácsi et al., 2009). Fessard and Bernard (2003) observed a slight increase in the percentage of apoptotic nuclei in cells exposed to 0.66 and 1 µg mL^{-1} of CYN for 24 h. Lankoff et al. (2007) reported slight though not significant induction of early apoptotic cells at concentrations 1 µg mL^{-1} and above after 21 h of exposure, and necrosis at doses of 1 and 2 µg mL^{-1}. The authors suggested that the relatively low level of apoptosis may result from the inhibition of protein synthesis by CYN. Gácsi et al. (2009) observed apoptosis at higher concentrations (5 and 10 µM corresponding to approximately 2.5 and 5 µg mL^{-1}, respectively after 18 h), and detected necrosis after longer exposure (24 and 48 h) to 10 µM (approximately 5 µg mL^{-1}) CYN. In HepG2 cells, several genes involved in the intrinsic as well as extrinsic apoptotic pathway were shown to be deregulated by CYN exposure at the transcriptional level, however it was not clear whether pro- or anti-apoptotic signals were prevailing (Štraser et al., submitted for publication).

In this study we measured three indicators of apoptosis: induction of caspase 3 and 7 activity, differential annexin V/PI staining and changes in MMP potential with the JC-1 probe. CYN had no influence on the activity of the effector caspases 3 and 7 and there was also no increase in the percentage of early and late apoptotic cells determined with the annexin V/PI staining. CYN also did not induce loss of MMP, which is often an early event of the apoptosis processes (Ly et al., 2003), but caused MHP. MHP appears to be one of the earliest changes associated with several apoptosis pathways (Nagy et al., 2007), however, it is a reversible switch and is not exclusively associated with apoptosis (Perl et al., 2004). It was on one hand reported to be an active step in apoptosis (Poppe et al., 2001) and on the other hand it was proposed as a step that inhibits apoptosis (Liang et al., 1999). It was hypothesized that it might serve a protective function against cell death by preserving Ca^{2+} ion homeostasis (Iijima, 2006). Thus, the pathophysiological role of MHP is currently not clear. From these results we conclude that CYN at concentrations up to 0.5 µg mL^{-1} and exposure up to 24 h in HepG2 cells does not induce apoptosis. The results corroborate the study with CYN exposed human peripheral blood lymphocytes (HPBLs), which showed that the ratio between the expression of the pro-apoptotic gene *BAX* and anti-apoptotic gene *BCL2* was in favor of *BCL2* (Žegura et al., 2011a), indicating that CYN may even suppress apoptosis.

5. Conclusion

Based on the results of the present study, we can conclude, that at non-cytotoxic concentrations oxidative stress does not play a substantial role in the genotoxicity of CYN in HepG2 cells. At concentrations that induce DNA damage CYN does not induce apoptosis, adding to its hazard, as cells with damaged DNA are not removed from the population, which may enhance the risk of mutations and consequently carcinogenesis.

Acknowledgments

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2.2 OSTALO POVEZOVALNO ZNANSTVENO DELO

2.2.1 Cilindrospermopsin ustavlja celični cikel in tako zniža celično proliferacijo pri celicah HepG2

Decreased cell-proliferation by cell-cycle arrest induced by cylindrospermopsin in HepG2 cells

Alja Štraser, Metka Filipič, Bojana Žegura

Marine Drugs (Special Issue "Compounds from Cyanobacteria"), v pregledu

Cianobakterijski citotoksin cilindrospermopsin (CYN) se vse pogosteje pojavlja v površinskih vodah po vsem svetu. Predstavlja potencialno nevarnost za ljudi pri kronični izpostavitvi, saj povzroča genotoksične učinke v številnih testnih sistemih in je potencialno karcinogen, vendar pa mehanizmi njegovega toksičnega in genotoksičnega delovanja niso dobro raziskani. V naši študiji smo pokazali, da CYN po podaljšanem času izpostavitve (72 h) povzroča nastanek dvoverižnih prelomov DNK (DSB) pri celicah humanega hepatoma HepG2. Rezultati kažejo, da CYN (0,1 – 0,5 µg/ml, 24 - 96 h) povzroča morfološke spremembe in znižuje živost celic v odvisnosti od koncentracije in časa. Pri testiranih pogojih nismo zaznali statistično značilnega povišanja puščanja laktat dehidrogenaze (LDH), kar kaže, da je znižana živost celic po izpostavitvi CYN posledica znižane celične rasti in ne smrti. To smo potrdili z imunocitokemijsko analizo proliferacijskega kazalca Ki67. Analiza celičnega cikla s pretočno citometrijo je pokazala, da CYN povzroča ustavitev cikla v G0/G1 fazi po 24 h in v S fazi po podaljšanem času (72 in 96 h) izpostavitve. Ti podatki podajajo nove dokaze, da je CYN direkten genotoksin, ki povzoča DSB, kar je potrebno upoštevati pri oceni tveganja za zdravje ljudi.

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Article

Double strand breaks and cell-cycle arrest induced by the cyanobacterial toxin cylindrospermopsin in HepG2 cells

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Abstract: The newly emerging cyanobacterial cytotoxin cylindrospermopsin (CYN) is increasingly being found in surface freshwaters, worldwide. It poses a potential threat to humans after chronic exposure as it was shown to be genotoxic in a range of test systems and is potentially carcinogenic. However the mechanisms of CYN toxicity and genotoxicity are not well understood. In the present study CYN induced formation of DNA double strand breaks (DSBs), after prolonged exposure (72 h), in human hepatoma cells, HepG2. CYN (0.1 - 0.5 µg/ml, 24 - 96 h) induced morphological changes and reduced cell viability in a dose and time dependant manner. No significant increase in LDH leakage could be observed after CYN exposure, indicating that the reduction in cell number was due to decreased cell proliferation and not due to cytotoxicity. This was confirmed by imunocytochemical analysis of the cell-proliferation marker ki67. Analysis of the cell-cycle using flow-cytometry showed that CYN has an impact on the cell cycle, indicating G0/G1 arrest after 24 h and S-phase arrest after longer exposure (72 and 96 h). Our results provide new evidence that CYN is a direct acting genotoxin, causing DSBs, and these facts need to be considered in the human health risk assessment.

Keywords: cylindrospermopsin, cell-cycle, cell-proliferation, double-strand breaks, HepG2 cells

1. Introduction

The cyanobacterial toxin cylindrospermopsin (CYN) is synthesized by a number of freshwater cyanobacterial species (for review see: [1]) and is increasingly being recognized as a potential threat to drinking water safety, worldwide. The toxin is a stable 415 Da tricyclic polyketide-derived alkaloid, containing a guanido group linked at C7 to hydroxymethyl uracil through a hydroxyl bridge [2]. CYN was first identified as the probable cause of a severe case of human poisoning in Australia in 1979 [3], and was since then found to be implicated in several cases of human intoxications and animal mortality [4-6]. It was first thought to be primarily associated with liver damage, but is now considered a cytotoxic and genotoxic toxin, due to its effects in other organs such as the kidneys, lungs, thymus, spleen, adrenal glands, intestinal tract, the immune system and the heart [4, 7, 8], and on DNA (for review see: [9]), respectively.

The toxin is a potent protein synthesis inhibitor [10-12], and contains several potential sites for reactivity that may form, protein and DNA adducts. There is evidence for its genotoxic activity *in vitro* [13-17] and *in vivo* [18, 19], and even carcinogenic potential of CYN has been indicated by preliminary results [20]. The majority of the studies show that CYN is a pro-genotoxin that needs to be activated by enzymes from the cytochome P450 (CYP450) family [13, 16, 17]. However despite of its apparent hazard, the mechanisms involved in CYN genotoxic and especially carcinogenic activity are poorly understood. Therefore the U.S. Environmental Protection Agency (EPA) classified CYN on the list of compounds with highest priority for hazard characterization [21]. The World Health Organisation (WHO) included CYN in the revision of the WHO "Guidelines for Drinking-water Quality, chemical hazards in drinking-water", but there is still insufficient information for the classification of CYN as a carcinogen by the International Agency for Research on Cancer (IARC).

Its protein synthesis inhibition ability and its genotoxic activity suggest that CYN has an impact on cell-proliferation and cell-cycle progression. The first response upon DNA damage is cell-cycle checkpoint activation, delaying cell-cycle progression and allowing cells to repair defects, thus preventing their transmission to the daughter cells [22]. Also the protein synthesis inhibition correlates with decrease in cellular proliferation and influences the onset and completion of mitosis [23-25]. Nevertheless limited data has been published regarding this topic in mammalian test systems. Therefore the aim of this study was to investigate the influence of CYN on cell-proliferation and cell-cycle progression in the metabolically active human hepatoma cell line, HepG2.

2. Results and Discussion

It is generally accepted that CYN is genotoxic as it induces DNA damage in several *in vitro* [13-17, 26] and *in vivo* test systems [18, 19]. In the present study the formation of DNA double strand breaks (DSBs) by CYN was shown for the first time. In addition the influence of genotoxic CYN concentrations on the cell-cycle and cell-proliferation in HepG2 cells was shown.

2.1 Viability of HepG2 cells after CYN exposure

CYN significantly affected cell viability in a dose and time dependant manner (Fig. 1, A). After 24 h of exposure, significant decrease in cell viability was detected at the concentration 0.3 µg/ml and above, however the cell survival at the highest tested concentration was still more than 70 %. After longer exposure (96 h), CYN reduced cell viability for about 50 to up to 65% at the concentrations 0.4 and 0.5 µg/ml, respectively. The toxin (0.5 µg/ml) induced morphological changes that were observed under the light microscope (Fig.1B) especially after longer exposure (from 48 h onwards).

There was no significant increase in LDH leakage in cells exposed to CYN at any of the tested time-points and concentrations, moreover a decrease in LDH leakage was observed. However total LDH content also decreased and was significantly different after 24 h (0.5 µg/ml), 48 h (0.25 and 0.5 µg/ml), 72 h (0.125 and 0.5 µg/ml) and 96 h (0.5 µg/ml) of exposure, again indicating decreased cell number. Therefore when calculating the ratio between LDH leakage and total LDH content in the sample (LDH leakage/total) the ratio remained at the control level (Fig. 2). These findings show that the reduced cell number after CYN exposure is not due to cytotoxicity but rather due to decreased cell proliferation. This correlates with our previous study on HepG2 cells, showing no apoptosis induction after CYN exposure [27]. Our results are also supported by the findings from Fessard and Bernard [28] and Lankoff et al. [29], who reported decrease in the number of mitotic figures and decrease in the mitotic index and proliferation in CHO-K1 cells exposed to CYN, respectively. Also in lymphoblastoid WIL2-NS cells exposed to CYN, a dose-dependent inhibition of cell division was observed [15], while in HepG2 cells [16] and in human peripheral blood lymphocytes (HPBLs) [17] CYN significantly decreased the nuclear division index.

Figure 1. CYN exposure reduces cell viability. HepG2 cells were incubated for 24, 48, 72 and 96 h with CYN (0.1, 0.2, 0.3, 0.4 and 0.5 µg/ml) and cell viability was assessed by the MTT assay. In each experiment a vehicle control (VC, 0.05 % methanol) was included. (A) Relative viability of cells is shown; the vehicle control was regarded as 100%. Significant difference between CYN-treated cells and the vehicle control is indicated by (*) ($p<0.05$), (**) ($p<0.01$) and (***) ($p<0.001$). (B) Micrographs of cells from the vehicle control group (VC) and cells exposed to 0.5µg/ml CYN under the microscope (magnified 200-times) at every experimental point. Independent experiments were performed in multiple replicates and were repeated at least three times.

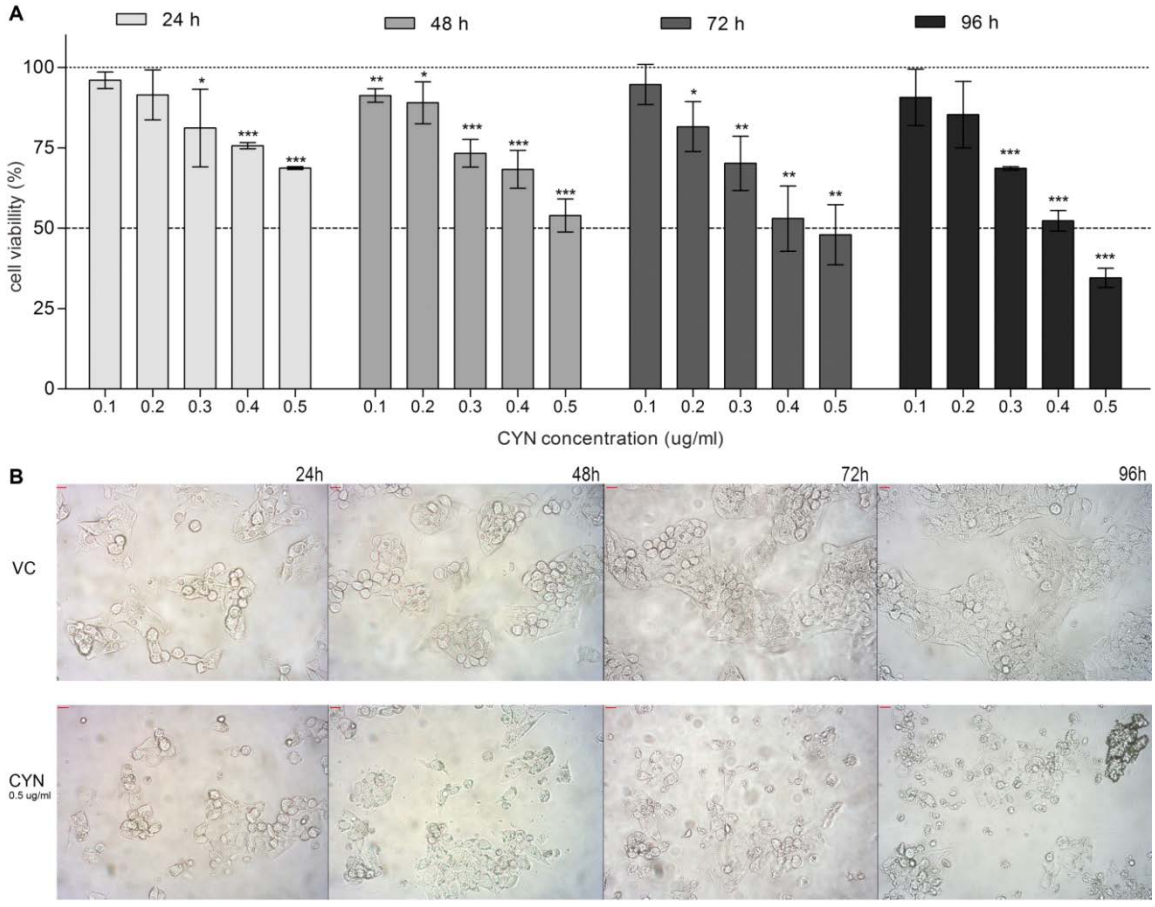
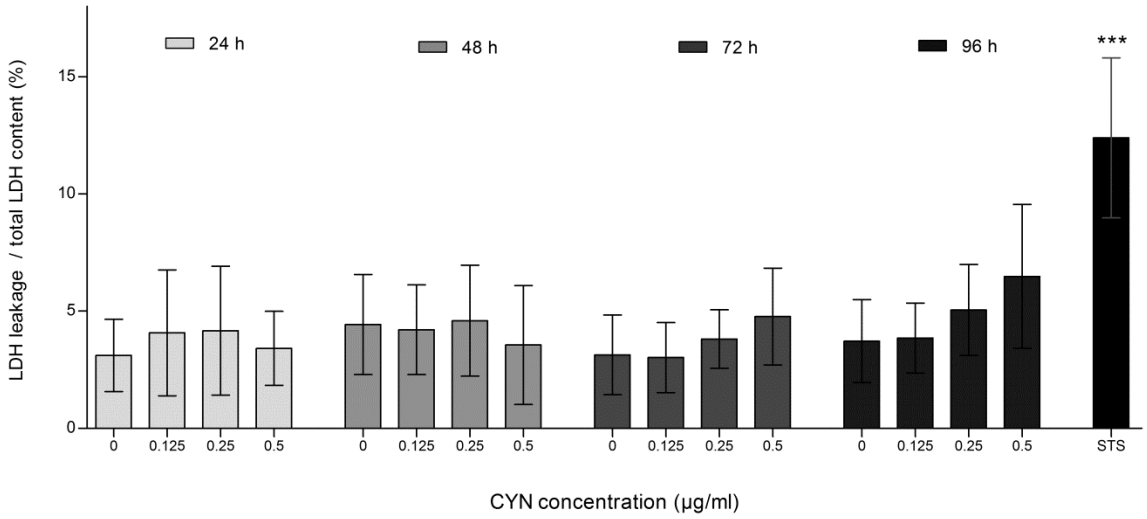


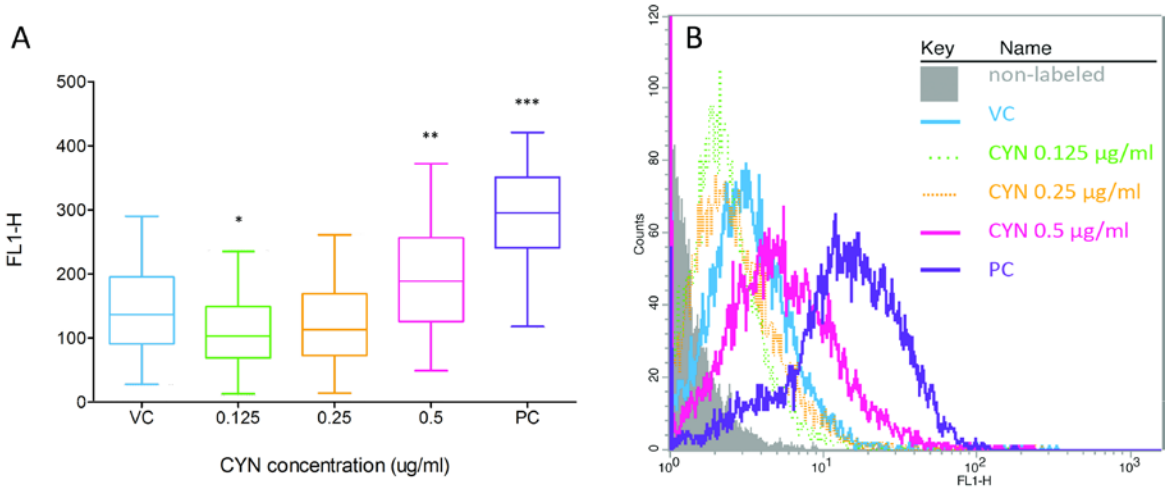
Figure 2. The influence of CYN on cell death. Cell death was assessed by lactate dehydrogenase (LDH) leakage. LDH content in the medium was determined spectrophotometrically using the Cytotox-ONE Homogenous Membrane Integrity Assay (Promega). LDH leakage after exposure to CYN (0.125, 0.25 and 0.5 µg/ml) for 24, 48, 72 and 96 h, and total LDH content at each experimental point was measured. In each experiment a vehicle control (VC, 0.05 % methanol) and a positive control (PC, 0.1 µM staurosporine) were included. The amount of the fluorescent product is proportional to the number of death cells. The percentage of LDH leakage from total LDH content is shown. Significant difference between CYN-treated cells and the vehicle control (VC) is indicated by (*) ($p<0.05$), (**) ($p<0.01$) and (***) ($p<0.001$). Independent experiments were performed in multiple replicates and were repeated at least three times.



2.2 Formation of DNA double strand beaks (DSBs) by CYN

DSBs are the most detrimental form of DNA damage as they can lead to chromosomal breakage and rearrangement [30]. CYN is expected to form DSBs as it was shown to be a clastogenic and aneugenic agent causing micronuclei *in vivo* and *in vitro* [13, 14, 16, 17, 19]. Induction of DSBs initiates fine-tuned networks that lead to repair by homologous recombination (HRR) or non-homologous end joining (NHEJ), checkpoint activation and cell-cycle arrest, apoptosis mostly via P53, activation of MAPKs, and the transcription factors AP-1 and NF-kB [31, 32]. Involvement of P53, AP-1 and NF-kB signalling in the cellular response to CYN was indicated in our previous study on the transcriptional response of HepG2 cells to CYN exposure [26]. CYN was also shown to deregulate several genes involved in DSB repair in HepG2 cells [26].

Figure 3. Induction of double strand breaks (DSB) by CYN. HepG2 cells were incubated for 72 h with CYN (0.125, 0.25 and 0.5 µg/ml) and the presence of DSB was analyzed by flow cytometry, indirectly through the detection of γH2A.X foci. In each experiment a vehicle control (VC, 0.05 % methanol) and a positive control (PC, 1µg/ml etoposide, 24 h) were included. (A) Distribution of the fluorescent signals of individual cells in the samples is shown. Data are presented as quantile box plots. The edges of the box represent the 25th and 75th percentiles, the median is a solid line through the box, and the bars represent 95% confidence intervals. In each sample 10^4 events were recorded and experiments were repeated three times. Significant difference between CYN-treated cells and the vehicle control (0) is indicated by (*) ($p<0.05$), (**) ($p<0.01$) and (***) ($p<0.001$). (B) Representative histograms are shown.

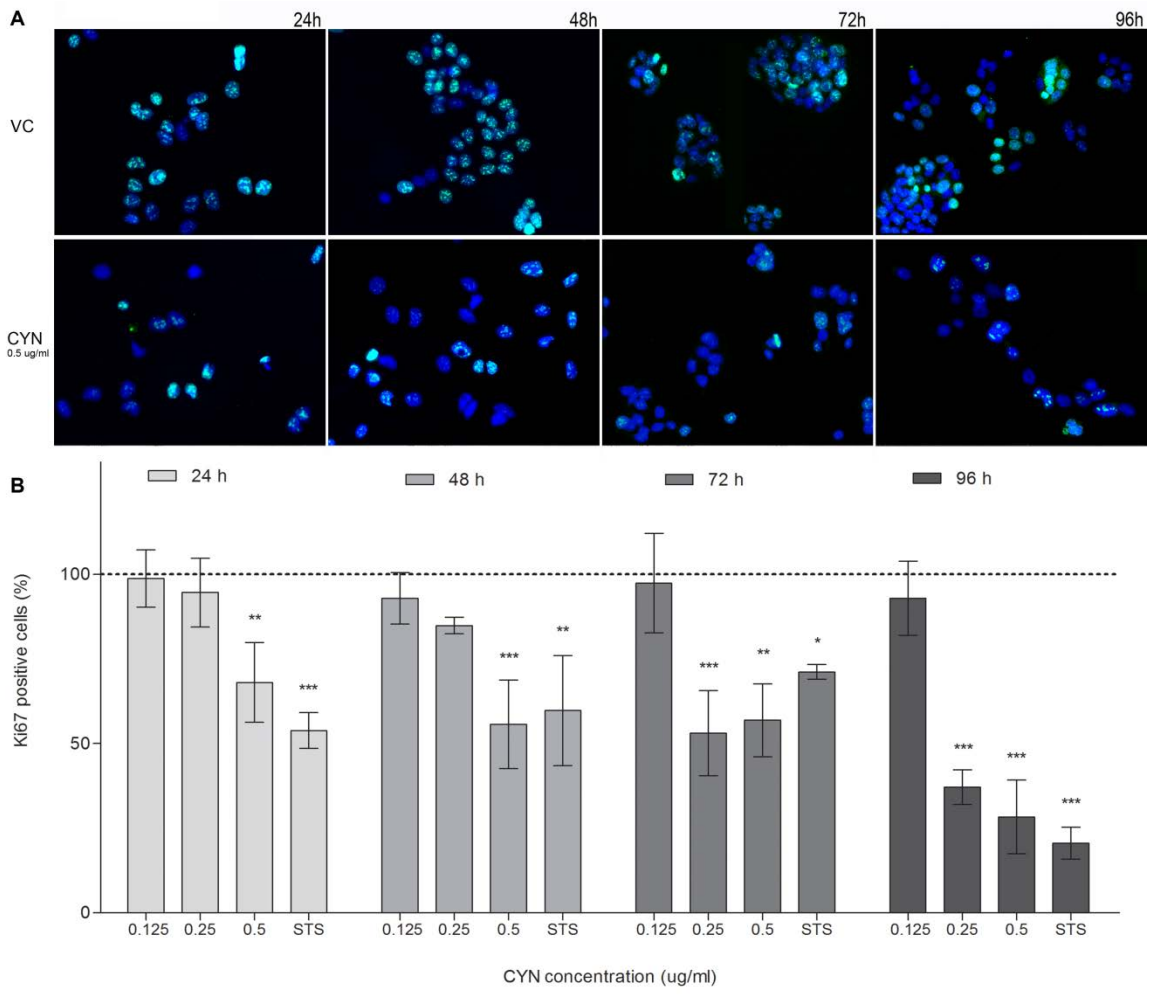


DSBs induction is rapidly followed by phosphorylation of the histone, H2AX [33], which is a component of the histone octomer in nucleosomes [34]. The phosphorylated H2AX histones (γH2AX) accumulate at sites of the DSBs, forming foci that correlate to DSBs within a 1 : 1 ratio [34, 35], and can therefore be used as a biomarker for DSBs and DNA damage. The presence of DSBs was analyzed by flow cytometry, by measuring the fluorescent signals of individual cells, indirectly through the detection of γH2AX foci. After 24 h of exposure there was no increase in DSB formation (data not shown), while after prolonged exposure (72 h) CYN significantly induced DSBs at 0.5 µg/ml (Fig. 3). At 0.125 µg/ml CYN significantly decreased the γH2AX signal compared to the control, the same was observed after 24 h of exposure to 0.125 and 0.25 µg/ml CYN (data not shown). This could be due to DSB repair processes or the protein synthesis inhibition by CYN; however it needs to be further elucidated.

2.3 Influence of CYN on cell proliferation

In addition to the measurement of cell viability and total LDH content the influence of CYN on cell proliferation inhibition was analysed by the detection of cells positive for the proliferation marker Ki67. CYN decreased the percentage of Ki67 positive cells at all exposure times (Fig. 4). After 24 and 48 h of exposure there was a statistically significant decrease at the highest tested concentration (0.5 µg/ml), while after longer exposure (72 and 96 h), the decrease in Ki67 positive cells was statistically significant already at 0.25 µg/ml. At the concentration 0.125 µg/ml CYN had no effect on the expression of Ki67 at any time point. The expression of the human Ki67 protein is strictly associated with cell proliferation as the protein is present during all active phases of the cell cycle (G1, S, G2, and M), and absent from resting cells (G0) [36]. Decrease in Ki67 positive cells could also be a direct consequence of CYN induced protein synthesis inhibition as reduction in Ki67 staining intensity after inhibition of protein synthesis was reported before [37]. However, as the Ki76 expression decrease correlated with the decrease in viable cells, and number of cells seen under the microscope as well as the total LDH content, it probably reflects reduced cell proliferation. Nevertheless, contribution of the protein synthesis inhibition to the decreased expression of Ki67 cannot be excluded.

Figure 4. CYN influence on Ki67 expression. HepG2 cells were incubated for 24, 48, 72 and 96 h with CYN (0.125, 0.25 and 0.5 µg/ml) and imunocytochemical staining of Ki67 was performed. In each experiment a vehicle control (VC, 0.05 % methanol) and a positive control (PC, 0.1 µM staurosporine) were included. 500 nuclei were counted under the fluorescent microscope and percentage of Ki67 positive cells was assessed (A). Significant difference between CYN-treated cells and the vehicle control (VC) is indicated by (*) ($p<0.05$), (**) ($p<0.01$) and (***) ($p<0.001$). Independent experiments were performed three times. (B) Representative fluorescent micrographs of the vehicle control (VC) and CYN (0.5 µg/ml) exposed cells are shown (magnified 460-times).

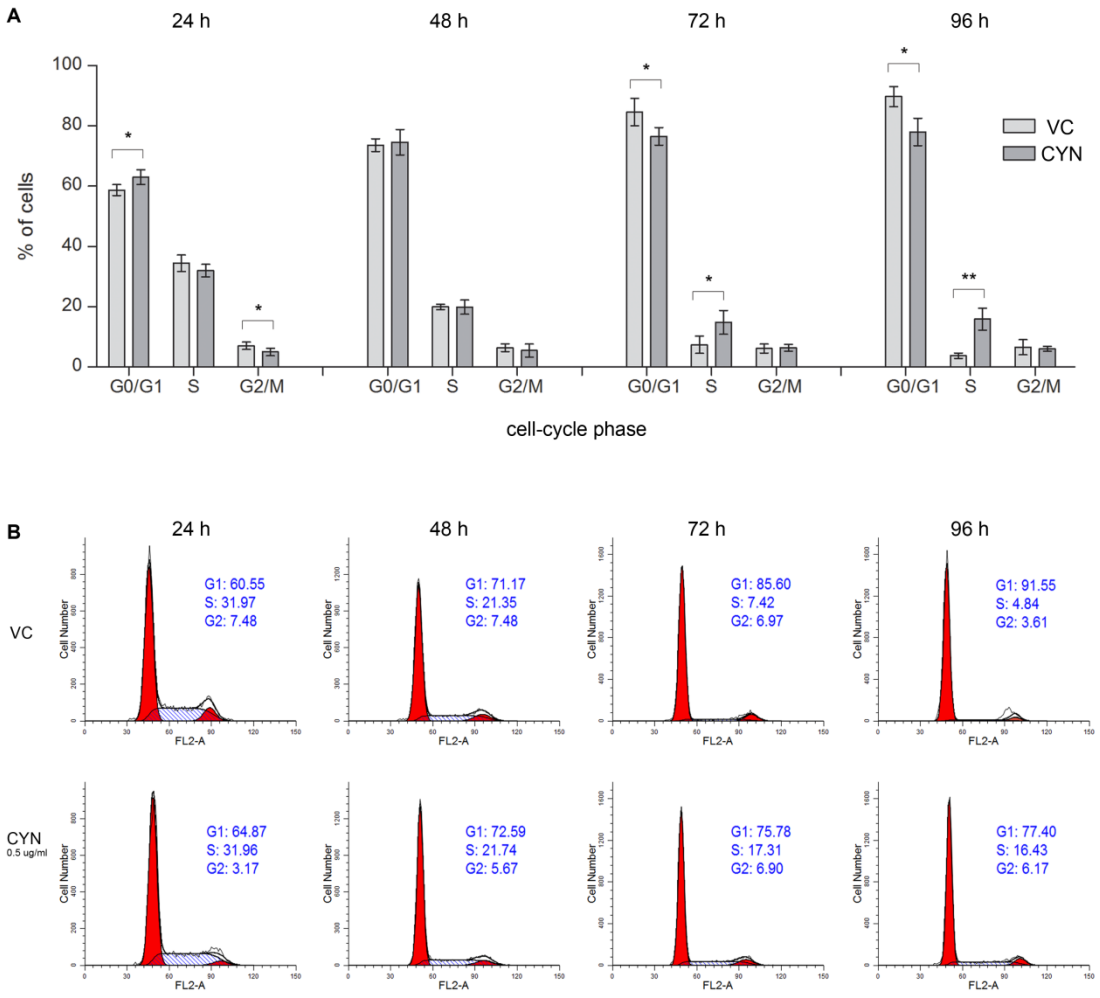


2.4 Influence on the cell-cycle progression

CYN (0.5 µg/ml) exposure affected the distribution of the cells through the cell-cycle (Fig. 5). After 24h CYN (0.5 µg/ml) significantly increased the amount of cells in G0/G1 phase and decreased the percentage of cells in G2/M phase, compared to the control population, indicating prevention of cells from entering S phase or even committing to cell division in the first place. It is well known that DNA damage can induce G1 phase arrest through P53 and CDKN1A (for reviews see [38, 39]). Induction of G1 phase arrest after CYN exposure (12 and 24 h) was also indicated by gene expression studies in HepG2 cells [26] and the involvement of P53 signalling and CDKN1A in the cellular response to CYN, supporting this findings, was already indicated by the up-regulation of CDKN1A and other P53 downstream regulated genes in HepG2 cells [16, 26, 40], in HPBLs [17] and human dermal fibroblasts (HDFs) [40]. Cell cycle arrest was also observed after 48 h although to a lesser extent and the changes were not statistically significant. Lower CYN concentrations (0.125 and 0.25 µg/ml) did not induce statistically significant changes in the cell-cycle distribution of cells, at any of the exposure times (data not shown). The positive control (STS 0.5 µM) significantly decreased the percentage of cells in the G0/G1 and increased the percentage of cells in the G2/M phase (G0/G1 = 35.24 ± 5.87; S = 27.88 ± 2.28; G2/M = 36.88 ± 3.59).

After longer exposure, the percentage of cells in S phase started to increase in a dose dependent manner and was significant after 72 and 96h exposure to 0.5 µg/ml CYN (Fig. 5). The increase of cells in S phase was accompanied by decrease in G0/G1 phase cells, indicating cell-cycle arrest by CYN in the S phase. There are three checkpoints in S phase: the replication checkpoint and the S/M checkpoint, which both respond to DNA replication errors, and the replication-independent intra-S-phase checkpoint that is induced in response to DNA double strand breaks (DSBs) [22]. It is assumed that CYN can bind to DNA as it contains sulphate, guanidine and uracil groups. Covalent binding of CYN or its metabolites to DNA in mice [18] and DNA strand breakage [19] have already been reported. In the present study the formation of DSBs after prolonged exposure to CYN was shown, which correlated with the time point of the S phase arrest. This observations are also supported by the results of our previous study where CYN deregulated several genes involved in nucleotide excision repair (NER), which repairs DNA adducts, and DSB repair genes in HepG2 cells [26]. Replication errors after CYN exposure were also indicated by several studies, that in addition to increased frequencies of micronuclei (MNi) [13, 15-17] reported appearance of other irregular DNA structures such as nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) [16, 17], arising from extrusions of either amplified DNA [41] or chromatin, whose replication has failed during S-phase [42] and DNA miss-repair, chromosome rearrangements or telomere end-fusions [43], respectively.

Figure 5. CYN influences cell-cycle phase distribution of the exposed cell population. HepG2 cells were incubated for 24, 48, 72 and 96 h with CYN (0.5 µg/ml) and cell-cycle analysis was performed by flow-cytometry using propidium iodide (PI) staining. In each experiment a vehicle control (VC, 0.05 % methanol) was included. The percentage of cells in G0/G1, S, and G2/M phases of the cell cycle were determined from FL2-A histograms using ModFit LT ™ (version 3.3). (A) Charts represent differences between cell distribution through the cell-cyle phases in CYN-treated cells and the vehicle control (VC), after 24, 48, 72 and 96 h. Significant difference is indicated by (*) ($p<0.05$) and (**) ($p<0.01$). In each sample 10^4 events were recorded and experiments were repeated three times. (B) Representative FL-2A histograms for the VC and CYN (0.5 µg/ml) are shown.



3. Experimental Section

3.1 Chemicals

Cylindrospermopsin (CYN) was from Enzo Life Sciences GmbH, Lausen, Switzerland. A 0.5 mg/ml stock solution of CYN was prepared in 50% methanol. William's medium E, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethylsulphoxide (DMSO) and staurosporine (STS) were obtained from Sigma, St. Louis, USA. Penicillin/streptomycin, foetal bovine serum (FBS), L-glutamine and phosphate buffered saline (PBS) were from PAA Laboratories, Dartmouth, USA. Trypsin was from BD-Difco, Le Pont-De-Claix Cedex, France. Mouse monoclonal IgG1, Anti-phospho-Histone H2A.X (Ser139), FITC conjugate, were from Milipore, Billerica, Massachusetts, USA. Alexa Fluor® 488 Goat Anti-Rabbit antibodies were from Invitrogen™, Life Technologies, Carlsbad, California, USA. Rabbit anti -Ki67 polyclonal antibodies were from Abcam®, Cambridge, UK. All other chemical reagents were of the purest grade available and all solutions were made using Milli-Q water.

3.2 Cell culture

HepG2 cells were a gift from Dr. Firouz Darroudi (Leiden University Medical Centre, Department of Toxicogenetics, Leiden, The Netherlands). The cells were grown at 37°C and 5 % CO2 in William's medium E containing 15 % foetal bovine serum, 2 mM l-glutamine and 100 U/ml penicillin/streptomycin.

3.3 Cell viability – MTT assay

Cell viability after exposure to CYN was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) according to Mosmann [44] with minor modifications [45]. The HepG2 cells were seeded onto 96-well microplates (Nunc, Naperville IL, USA) at a density of 3000 cells/well and incubated for 4 h at 37°C in 5 % CO2 to attach. Fresh medium containing CYN was added to the wells to gain final concentrations of 0.1, 0.2, 0.3, 0.4 and 0.5 μg/ml. In each experiment a vehicle control (0.05 % methanol) was included. Measurements were taken after 24, 48, 72 and 96 h after the exposure to CYN. Images of control and exposed cells were taken under the light microscope (Nikon, Diaphot) at each experimental point. Independent experiments were performed in 5 replicates and were repeated 3- times.

3.4 Lactate Dehydrogenase (LDH) Leakage

Lactate Dehydrogenase (LDH) Leakage was determined using CytoTox-ONE™ Homogeneous Membrane Integrity Assay. Cells were seeded on black 384 well plates

(Corning Costar Corporation, Corning, NY, USA) at the density of 3000 cells/well in 4 replicates. After incubation at 37°C in 5 % CO2 for 24 h, the growth medium was replaced with fresh medium containing 0.125, 0.25 and 0.5 µg/ml CYN and cells were exposed to CYN for 24, 48, 72 and 96 h. In each experiment, a vehicle control (0.05 % methanol) and a positive control (0.1 µM STS) were included. After the exposure the assay was performed according to the manufacturers' protocol with minor modifications. In brief cells were left to cool to room temperature for 20 min and 25 µl of the CytotoxOne reagent was added to each well. The cells were shaken for 30 s and incubated for 10 min at room temperature. After that, 12.5 µl stop solution was added to each well and after shaking for 10 s the fluorescence was measured at 560 nm excitation and 590 nm emission, gain 55, using a microplate reading spectrofluorimeter (SynergyMx, BioTek). Differences in cell number and growth after the exposure to CYN were assessed by parallel performance of the maximum LDH release control, determining total LDH content after total lysis of the cells, as described by the manufacturer. The maximum LDH release control was performed in the same way as the LDH leakage assay, with the sole difference that 0.5 µl of lysis solution was added in each well before the CytotoxOne reagent addition. The fluorescence was measured at 560 nm excitation and 590 nm emission, gain 55, using a microplate reading spectrofluorimeter (SynergyMx, BioTek). Independent experiments were performed in 5 replicates and were repeated 2-times.

3.5 DSB detection – H2AX foci analysis

The cells were seeded on T25 flasks at the density of 0.8 x 10^6 per plate, left to attach overnight and exposed to CYN (0.125, 0.25 and 0.5 µg/ml) for 24 (short time exposure) and 72 h (long time exposure). In each experiment a vehicle control (0.05 % methanol) and a positive control (1 µg/ml etoposide, 24 h) were included. At the end of the exposure floating and adherent cells were collected by trypsinization. For the fixation the cells were centrifuged at 800 rpm, 4°C for 5 min, washed twice with ice cold PBS, resuspended in 0.5 ml cold PBS and ethanol (1.5 ml) was added drop wise into the cell pellet, while vortexing. The cells were fixed at 4°C overnight and stored at -20°C till analysis. Fixed cells were centrifuged at 1200 rpm for 10 min, washed twice with ice cold 1x PBS, resuspended in 0.5 ml 1x PBS containing 2000-fold diluted anti-γH2AX antibodies, mixed and incubated at 4 °C for 30 min in the dark. Labelled cells were then washed twice and resuspended in 0.3 ml of 1x PBS. Flow cytometric analysis was carried out on a FACSCalibur flow cytometer (BD Biosciences PharmingenTM, San Diego, CA, USA). FITC intensity, corresponding to DSBs, was detected in the FL1-H channel. In each sample 10^4 events were recorded. Independent experiments were repeated 3- times. For further analysis the raw data (FITC intensities of each cell, FL1-H intensity), obtained from the CellQuest Pro software (BD Biosciences), was converted from the .fcs to the .csv format, using the

program FCSExtract, which is available on the website: http://research.stowers-institute.org/efg/ScientificSoftware/Utility/FCSExtract/index.htm.

3.6 Cell-proliferation - immunocytochemical staining of the proliferation marker Ki67

Cell-proliferation after CYN exposure was analysed as described by Hreljac et al. [46], with minor modifications. Cells were seeded onto 24 well plates (Corning Costar Corporation, Corning, NY, USA), containing poly-L-Lysine slips, at the density of 30000 cells/well, left to attach overnight and exposed to 0.125, 0.25 and 0.5 μg/ml CYN for 24, 48, 72 and 96 h. In each experiment, a vehicle control (0.05 % methanol) and a positive control (0.1 μM STS for 24 h) were included. The cells were fixed at room temperature (RT) in 100% methanol for 10 min, permeabilized with 0.1% Triton-X for 10 min, and blocked with 4% BSA for 15 min. Polyclonal anti-Ki67 rabbit antibodies, diluted 1:500 in PBS, were added to the cells and incubated for 1 hr. Secondary anti-rabbit Alexa-Fluor 488 antibodies, diluted 1:500, were added and incubated for 90 min, all at RT. Hoechst (diluted 1:1000) was added for 5 min to stain the nuclei. Between the steps the slides were washed 5-times in PBS. After the staining anti-fade reagent was added on the object-slides and the slips were mounted on them and sealed. Slides were kept at -20°C until scoring. Cells were scored under a fluorescence microscope (Nicon Eclipse Ti); pictures were obtained with FLoid® Cell Imaging Station (Life technologies). Ki67 positive nuclei were granularly stained with intense green fluorescence. The percentage of proliferating cells was calculated as the ratio between Ki67 positive nuclei and total nuclei (stained with Hoechst). 500 cells were scored for each experimental point. Independent experiments were repeated 3-times.

3.7 Cell-cycle analysis by flow cytometry

HepG2 cells were seeded at a density of 400 000 cells/well into 6-well plates (Corning Costar Corporation, Corning, NY, USA). After incubation at 37°C in 5 % CO2 for 24 h, the growth medium was replaced with fresh medium containing 0.125, 0.25 and 0.5 μg/ml CYN and incubated for 24, 48, 72 and 96 h. In each experiment, a positive control (1 μM STS for 24 h) and a vehicle control (0.05 % methanol) were included. After the exposure, cells were harvested and fixed as described above (3.5 DSB detection – H2AX foci analysis). Fixed cells were centrifuged at 1200 rpm for 10 min, washed twice with ice cold 1x PBS and stained with 0.5 ml propidium iodide/RNAse staining buffer for 15 min at room temperature according to the manufacturer's recommendations. Flow cytometric analysis was carried out on FACSCalibur (BD Biosciences PharmingenTM, San Diego, CA, USA). Changes in the distribution of cells through the phases of the cell cycle were analyzed in the FL2 channel, where 10^4 events were recorded for each sample. The

percentage of cells in G0/G1, S, and G2/M phases of the cell cycle were determined from FL2-A histograms using ModFit LT ™ (version 3.3, for Windows 7) Verity Software House, Topsham, Maine, USA. Analysis was performed on single cells, by elimination of cell aggregates by gating FL2-W versus FL2-A. Independent experiments were repeated 3-times.

3.8 Statistical analysis

Statistical significance between treated groups and the control was determined by One-way analysis of variance and Dunnett's Multiple Comparison Test, using GraphPad Prism 5 (GraphPad Software). For the γH2AX foci analysis the statistical significance between treated groups and the vehicle control was determined with a linear mixed-effects model. Calculations were done with the statistical program R [47] and its packages reshape [48], ggplot2 [49] and nlme [50].

4. Conclusions

Based on the results of the present study, we can conclude, that CYN is genotoxic for HepG2 cells and potentially presents a serious health risk for humans, as the toxin induced DNA double strand breaks at non-cytotoxic concentrations and reduced cell-proliferation of HepG2 cells by induction of cell cycle arrest in G0/G1 phase after 24 h of exposure and in S phase after prolonged exposure (72 and 96 h).

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Conflict of Interest

The authors report no conflict of interest.

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2.2.2 Genotoksično delovanje cianobakterijskega pentapeptida nodularina na celice HepG2

Genotoxic effects of the cyanobacterial pentapeptide nodularin in HepG2 cells

Alja Štraser, Metka Filipič, Irena Gorenc, Bojana Žegura

v pripravi

Cianobakterijski pentapeptid nodularin (NOD) je močan zaviralec proteinskih fosfataz PP1 in PP2A in povzroča pogine živali. Študije, ki so na voljo, kažejo, da je NOD potencialen ne-genotoksičen karcinogen. Analizirali smo genotoksično delovanje NOD na celični liniji humanega hepatoma HepG2, z zaznavanjem nastanka oksidativnih poškodb DNK z modificiranim testom komet z encimom formamidopirimidin glikozilaza (Fpg). Povišan nastanek reaktivnih kisikovih zvrsti (ROS) po izpostavljenosti NOD smo analizirali z uporabo sonde DCFH-DA. Poleg tega smo analizirali spremembe v izražanju genov iz poti P53, vključene v odziv na poškodbe DNK (*P53*, *CDKN1A*, *GADD45α*, *MDM2*) in apoptozo (*BAX* in *BCL2*) s kvantitativnim PCR v realnem času. NOD je povzročal od koncentracije in časa odvisno povišanje ROS. Necitotoksične koncentracije NOD so povzročile le rahlo povišanje poškodb DNK. Vendar pa so nastale znatne statistično značilne oksidativne poškodbe DNK. Največ oksidativnih poškodb smo zaznali po 4 h izpostavitve. Opazili smo le manjše spremembe v izražanju genov, kar ni potrdilo, da NOD sproži signalizacijo P53. Naši rezultati nakazujejo, da je NOD posredno genotoksičen in deluje preko povišanja ROS že pri nizkih, necitotoksičnih, za okolje pomembnih koncentracijah.

GENOTOXIC EFFECTS OF THE CYANOBACTERIAL PENTAPEPTIDE NODULARINE IN HepG2 CELLS

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Abstract:
The cyanobacterial pentapeptide nodularin (NOD) is a potent inhibitor of protein phosphatases PPA1 and PPA2, and causes animal mortality. The few studies available indicate that NOD is a potential non-genotoxic carcinogen. We evaluated NOD genotoxicity in the human hepatoma cell line, HepG2, analyzing the induction of oxidative DNA damage with the modified comet assay, using the enzyme formamidopyrimidine glycosilase (Fpg). Reactive oxygen species (ROS) production after NOD exposure was analysed using the DCFH-DA probe. In addition, changes in the expression of genes from the P53 pathway, involved in the response to DNA damage (*P53*, *CDKN1A*, *GADD45α*, *MDM2*) and apoptosis (*BAX* and *BCL2*) were determined, using quantitative real-time PCR. NOD induced time and dose dependant increase in ROS production. Non-cytotoxic concentrations of NOD induced substantial oxidative DNA damage, with the highest level observed after 4 h of exposure. Only slight changes in gene expression were observed that could not confirm induction of the P53 network by NOD. Our results provide new evidence that NOD genotoxic effects are mediated through ROS production, already at low, non-cytotoxic and environmentally relevant concentrations.

Keywords:
Nodularin, HepG2, oxidative DNA damage, reactive oxygen species

Abbreviations:
NOD – Nodularin; MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI – propidium iodide; ROS – reactive oxygen species;

1 Introduction:

The monocyclic pentapeptide nodularin (NOD) is along with the microcystins (MCs), the most frequently occurring and widespread cyanotoxin in brakish and fresh-water blooms, but is in contrast to the MCs not as extensively studied. Only 9 analogue specimens of NODs (one nontoxic) have been identified so far (Rzymski, Poniedziałek et al. 2011). The congener NOD (Fig. 1) seems to be the main component of environmental samples, with other variants occurring rarely and in negligible concentrations (Sivonen, Kononen et al. 1989; Jones, Blackburn et al. 1994). The sole freshwater cyanobacterial source of NODs appears to be the cyanobacterial species *Nodularia spumigena*, which is spread across the world, preferably inhabiting brackish and estuarine environments (Pearson, Mihali et al. 2010). NOD is similar in structure to MCs and has similar mechanisms of action. It contain the unusual C20 amino acid ADDA ((2S, 3S, 8S, 9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-diene acid) (Sivonen and Jones 1999), which is found only in cyanobacterial peptides and is thought to be responsible for the bioactivity of these compounds.

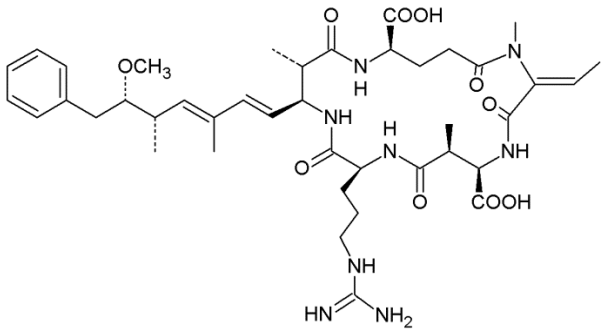

Fig. 1 chemical structure of nodularin (NOD).

The principal mechanism of the toxicity of these toxins is the specific inhibition of eukaryotic protein serine/threonine phosphatases 1 and 2A (PP1 and PP2A) *in vitro* (Runnegar, Andrews et al. 1987; Eriksson, Toivola et al. 1990; Honkanen, Zwiller et al. 1990; MacKintosh, Beattie et al. 1990) and *in vivo* (Runnegar, Kong et al. 1993), the consequence of which is hyperphosphorylation of cellular proteins and thereby disruption of many cellular processes, alteration and rearrangement of the cytoskeleton, loss of cell-cell adhesion, and consequently disruption of the hepatic architecture, leading to intrahepatic hemorrhage and hepatic insufficiency (for review see: (Chorus and Bartram 1999; Duy, Lam et al. 2000; Hjørnevik, Fismen et al. 2012)). NOD has been implicated in several animal deaths, causing massive liver hemorrhage (Pearson, Mihali et al. 2010), but fortunately no cases of human intoxications have been reported so far. Nevertheless the toxin bioaccumulates and has been found in tissues of clams, shrimps and fish – all used for consumption (Van Buynder, Oughtred et al. 2001; Pearson, Mihali et al. 2010; Stewart, Eaglesham et al. 2012) and is not destroyed by cooking (Van Buynder, Oughtred et al. 2001). Therefore chronic exposure of humans to low doses of NOD is possible and is of

great concern as it is a potential human carcinogen. NOD is considered to act predominantly as a tumor promoter through the protein phosphatase inhibition; however evidence is accumulating that it is also genotoxic (for review see: (Žegura, Štraser et al. 2011)). The similarity of this toxin to microcystin-LR (MCLR), suggests that it poses at least the same risks to human health. On the contrary to MCLR, which has been classified in group 2b (possible carcinogen for humans) by the IARC, NOD has been classified as an »animal carcinogen but not classifiable as to its carcinogenicity to humans« (group 3), predominantly due to the lack of sufficient experimental data (IARC 2010). Therefore information on the genotoxicity and potential carcinogenicity of NOD is urgently needed.

The aim of this study was to investigate the genotoxic properties of NOD in the human hepatoma cell line, HepG2, using the comet assay, the modified comet assay, detection of intracellular ROS production and toxicogenomic analysis.

2 Materials and methods:

2.1 Chemicals

Nodularin (NOD) was from Enzo Life Sciences GmbH, Lausen, Switzerland. A 1 mg/ml stock solution of CYN was prepared in 50% methanol. William's medium E, glycogen, ethylenediaminetetraacetic acid (EDTA), benzo[a]pyrene (BaP), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), tert-butyl hydroperoxide (t-BOOH) and dimethylsulphoxide (DMSO) were obtained from Sigma, St. Louis, USA. Penicillin/streptomycin, foetal bovine serum (FBS), L-glutamine and phosphate buffered saline (PBS) were from PAA Laboratories, Dartmouth, USA. Trypsin was from BD-Difco, Le Pont-De-Claix Cedex, France. All other chemical reagents were of the purest grade available and all solutions were made using Mill-Q water. Triton X-100 was from Fisher Sciences, New Jersey, USA. Ethidium-bromide solution was from Promega, Madison, Wisconsin, USA. TRIzol reagent, normal melting point agarose (NMP), low melting point agarose (LMP) and 2',7'-dichlorfluorescein-diacetate (DCFH-DA) were from Invitrogen, Paisley, Scotland. Formamidopyrimidine glycosylase (Fpg) was a gift from Dr. Andrew R. Collins (Department of Nutrition, Faculty of Medicine, University of Oslo, Norway) and concentrations of the enzyme were prepared according to his protocol. cDNA High Capacity Archive Kit, TaqMan Universal PCR Master Mix and the Taqman Gene Expression Assays were from Applied Biosystems, New Jersey, USA. All other chemical reagents were of the purest grade available and all solutions were made using Mill-Q water.

2.2 Cell culture

HepG2 cells were a gift from Dr. Firouz Darroudi (Leiden University Medical Centre, Department of Toxicogenetics, Leiden, The Netherlands). The cells were grown at 37°C and 5% CO_2 in William's medium E containing 15% foetal bovine serum, 2mM l-glutamine and 100 U/ml penicillin/streptomycin.

2.3 Cell viability – MTT assay

Cell viability after exposure to NOD was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) according to Mosmann (Mosmann 1983) with minor modifications as described before (Štraser, Filipič et al. 2011). Cells were exposed to NOD 0, 0.01, 0.05, 0.1, 0.5, 1, 5 and 10 μg/ml for 24 h.

2.4 Intracellular ROS formation

The formation of intracellular ROS was measured using a fluorescent probe, DCFH-DA as described by Osseni et al. (1999) (Osseni, Debbasch et al. 1999), with minor modifications (Zegura, Lah et al. 2004). In brief, after 24 h of incubation at 37° C in 5% CO_2, cells were incubated with 20 μM DCFH-DA. After 30 min, DCFH-DA was removed and cells were treated with NOD (0, 0.01, 0.1 and 1 mg/ml) in PBS. For kinetic analyses the dishes were maintained at 37° C and the fluorescence intensity was determined every 30 min using a microplate reading spectrofluorimeter (Tecan, Genios) at the excitation wavelength of 485 nm and the emission wavelength of 530 nm.

2.5 Comet assay

HepG2 cells were seeded at a density of 60 000 cells/ml into 12-well plates (Corning Costar Corporation, Corning, NY, USA). After incubation at 37°C in 5 % CO_2 for 20 h, the growth medium was replaced with fresh medium containing 0, 0.01, 0.1 and 1 μg/ml NOD and incubated for 4, 12 and 24 h. To examine the levels of oxidative damage, the modified comet assay was used as described by Collins et al. (1993) (Collins, Duthie et al. 1993), with minor modifications (Zegura, Sedmak et al. 2003). In brief, after the lysis the slides were washed three times for 5 min with endonuclease buffer (40 mM HEPES-KOH, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/ml bovine serum albumin, pH 8.0). Fifty microlitre aliquots formamidopyrimidine glycosylase (Fpg) were added, covered with a cover glass and incubated at 37°C for 45 and 30 min, respectively. The control slides received only enzyme buffer. The slides were then processed as described earlier.

2.6 Gene expression

Cells were seeded on T-25 flasks (Corning Costar Corporation, Corning, NY, USA) at a density of 10^6 cells/flask and incubated for 24 h at 37°C and 5 % CO2. They were exposed to NOD (0, 0.01, 0.1 and 1 μg/ml) and incubated for 4, 12 and 24 h. In each experiment, a positive control (30 μM BaP) and a vehicle control (0.05 % methanol) were included. RNA was isolated with TRIzol® Reagent, quantified on a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA) and reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit. All procedures were performed according to the manufacturer's instructions. Gene expression of *P53*, *MDM2*, *GADD45A*, *CDKN1A*, *BAX* and *BCL* was quantified using real-time quantitative PCR (ABI 7900 HT Sequence Detection System, Applied Biosystems, USA). TaqMan Universal PCR Master Mix and the following Taqman Gene Expression Assays

were used (all from Applied Biosystems): *P53* (tumor protein p53), Hs00153349_m1; *MDM2* (Mdm2, 'transformed 3T3 cell double minute 2', p53 binding protein gene), Hs00234753_m1; *GADD45A* (‘growth arrest and DNA damage-inducible gene, alpha'), Hs00169255_m1; and *CDKN1A* ('cyclin-dependent kinase inhibitor 1A'), Hs00355782_m1; *BAX* (BCL2 associated X protein), Hs99999001_m1; *BCL2* (B-cell CLL/lymphoma 2), Hs00608023_m1. Amplification of GAPDH probe was performed as an internal control. The conditions for PCR were 50°C for 2 min, 95°C for 10 min and 40 cycles at 95°C for 15 s and 60°C for 1 min. The data obtained from Taqman Gene Expression Assays were analyzed using the ΔΔCt algorithm. The expression levels of target mRNAs were normalized to the GAPDH mRNA level. Two independent experiments were performed each time in two parallels.

2.7 Statistical analysis

The statistical analyses were performed with GraphPad Prism 5 software. For the comet assay one-way analysis of variance (ANOVA) was used to analyze the differences between treatments within each experiment. Dunnett’s test was used for multiple comparison versus the control; $P<0.05$ was considered as statistically significant (*). Difference between samples in the MTT test, the ROS detection, and for the gene expression statistical significance between treated groups and controls was determined by Two-tailed Student t-test comparison of the mean and $P<0.05$ was considered significant. Independent experiments were preformed in multiple replicates and were repeated at least three times.

3 Results:

3.1 NOD influence on viability of HepG2 cells

The HepG2 cells were exposed to 0.01, 0.1 and 1 µg/ml of NOD for 24 h to assess the cytotoxicity of NOD. None of the tested concentrations of CYN significantly affected the viability of HepG2 cells. Therefore concentrations of up to 1 µg/ml were used for further experiments.

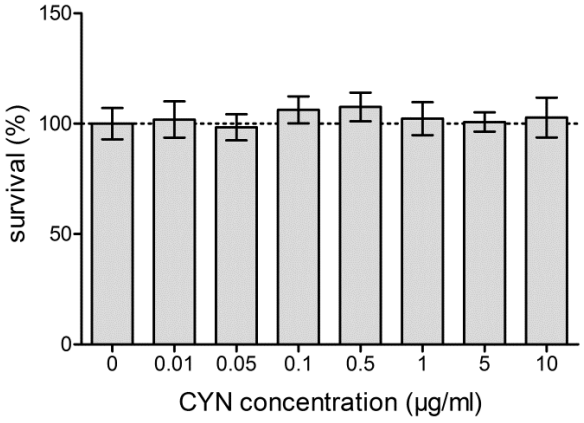

Fig. 2 The effect of NOD on the viability of HepG2 cells. The viability was determined with the MTT assay after the exposure to different concentrations of NOD (0.01 - 10 µg/ml) for 24 h.

3.2 Induction of reactive oxygen species (ROS)

To explore whether NOD increases the level of ROS in HepG2 cells, the fluorescent probe DCFH-DA was used. The probe is hydrolyzed by intracellular esterases to a non-fluorescent product. In the presence of ROS it is rapidly oxidized to highly fluorescent 2',7'-dichlorofluorescein (DCF). The DCF fluorescence intensity is proportional to the amount of ROS formed intracellularly. H_2O_2 is the principle ROS responsible for the oxidation of DCFH-DA to DCF (LeBel, Ischiropoulos et al. 1992). A dose dependent statistically significant increase of DCF fluorescence intensity was observed in cells treated with 0.01, 0.1 and 1 µg/ml NOD (Fig. 3), which steadily increased with incubation time. After 5 h incubation, the fluorescence intensity at the highest dose of NOD used was about 2.5 times higher than in the control cells, and the positive control (0.5 mM t-BOOH) was about 7.3 times higher. DCF fluorescence intensity increased slightly up to 5 h also in the control cells, suggesting that ROS were also formed in non-treated HepG2 cells under the experimental conditions used.

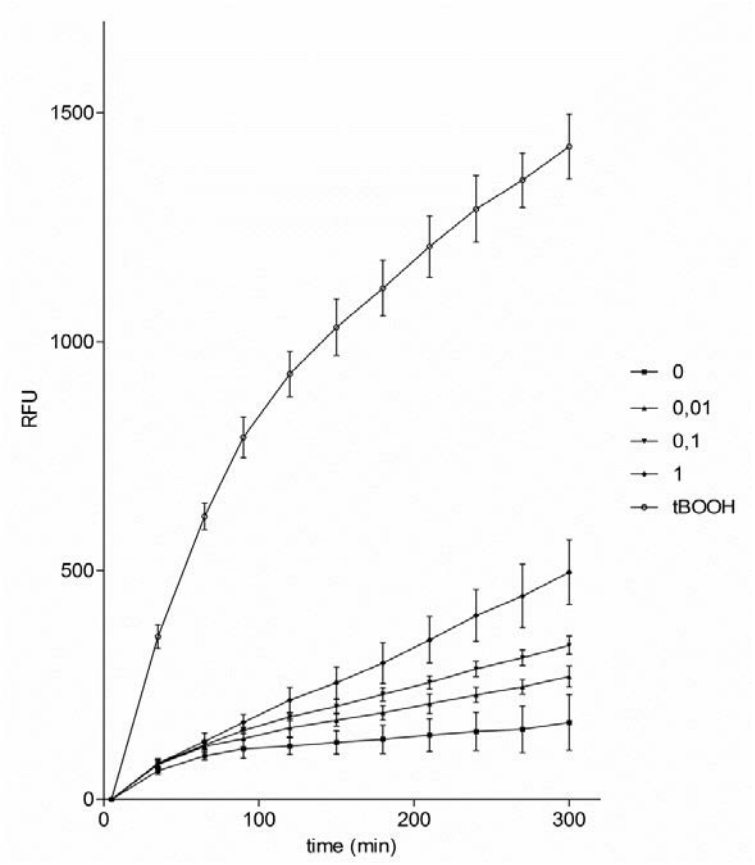

Fig 3. NOD-induced increase of DCF fluorescence in HepG2. The HepG2 cells were pretreated with DCFH-DA (20 µM) for 30 min, washed and then exposed to different concentrations of NOD (0, 0.01, 0.1 and 1 µg/ml). NOD concentrations that induced significant increase in DCF-fluorescence compared to the vehicle control (0) are shown. Tert-butyl hydroperoxide (0.5 mM t-BOOH) was used as the positive control (PC). DCF fluorescence intensity was measured at 30 min intervals during the 5 h incubation.

3.3 Induction of oxidized purines

Induction of oxidized purines was studied using the modified comet assay with the purified DNA damage specific enzyme, Fpg, which recognizes and excises oxidized purines (Collins, Dusinska et al. 1996). After 4 h of exposure NOD significantly increased the amount of Fpg sensitive sites at all concentrations tested, while no DNA damage increase was observed without Fpg digestion (Fig. 5). After 12 and 24 h of exposure the amount of Fpg-sensitive sites decreased and was significant at the concentrations 0.1 and 1 µg/ml. There was also minor DNA damage without Fpg digestion at these time points and concentrations, but was more pronounced after the enzyme digestion. These results indicate that DNA damage caused by NOD is probably caused by oxidative stress.

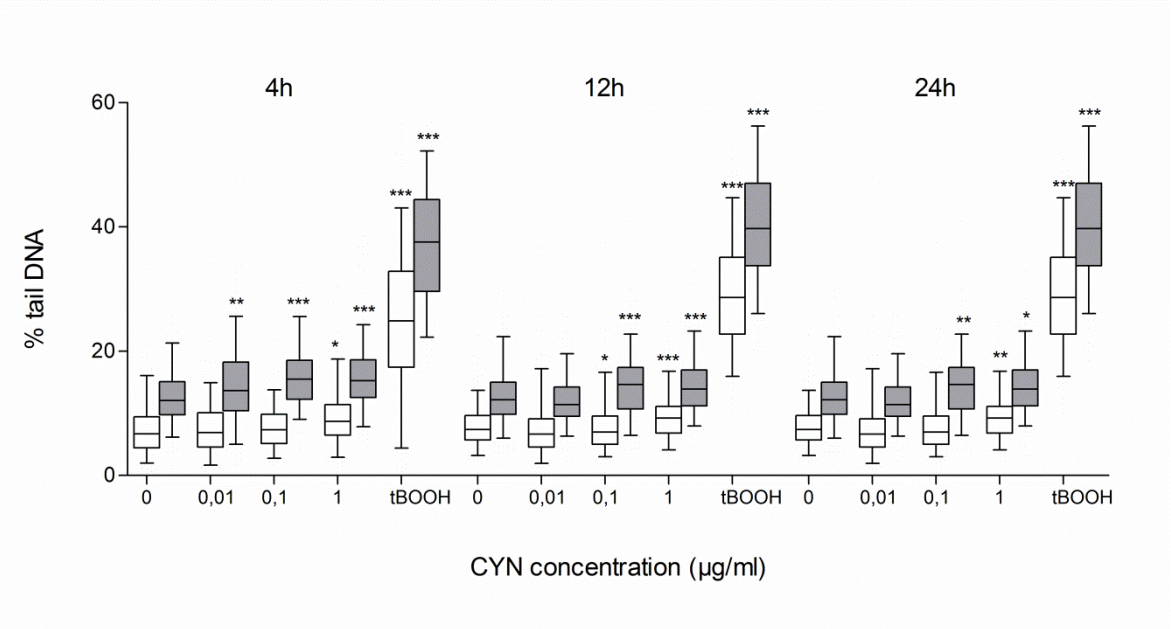

Fig. 4 The level of NOD-induced DNA strand breaks without (white box plots) and with formamidopyrimidine glycosylase (Fpg) digestion (gray box plots). The cells were exposed to NOD (0, 0.01, 0.1 and 1 µg/ml) for 4, 12 and 24 h, then the modified comet assay was performed. Tert-butyl hydroperoxide (0.5 mM) was used as the positive control (PC). The levels of DNA strand breaks and oxidised purines are expressed as percent of tail DNA (% tail DNA). Fifty cells were analyzed per experimental point in each of the three independent experiments. Data are presented as quantile box plots. The edges of the box represent the 25th and 75th percentiles, the median is a solid line through the box, and the error bars represent the 95% confidence intervals. Significant difference (1-way ANOVA; Dunnett's Multiple Comparison test) between NOD-treated cells and the vehicle control (0), and between Fpg digested and undigested samples is indicated by *P < 0.05, **P < 0.01, and ***P < 0.001.

3.4 Effect of NOD on changes in gene expression

The expression of selected DNA damage responsive genes and oxidative stress responsive genes were analyzed after 4, 12 and 24 h exposure of HepG2 cells to 0, 0.01, 0.1and 1 µg/ml of NOD by quantitative real-time PCR (Table 1).

Table 1: Changes in gene expression of genes involved in DNA damage response, oxidative stress response and apoptosis, after exposure of HepG2 cells to NOD (0.01 - 1 µg/ml) for 4, 12 and 24 h. The results are expressed as mRNA expression fold-change of the vehicle control (0) ± STD (ΔΔCt algorithm).

gene	CYN concentration (µg/ml)	4h	12h	24 h
P53	0	1.00 ± 0.08	1.00 ± 0.06	1.01 ± 0.14
	0.01	0.95 ± 0.08	1.07 ± 0.05	0.95 ± 0.04
	0.1	0.84 ± 0.12	1.04 ± 0.03	0.91 ± 0.07
	1	0.83 ± 0.13	0.98 ± 0.02	0.84 ± 0.06
	PC	0.62 ± 0.15	1.10 ± 0.05	0.94 ± 0.09
CDKN1A	0	1.01 ± 0.12	1.00 ± 0.02	1.01 ± 0.14
	0.01	0.80 ± 0.07**	1.06 ± 0.04	1.21 ± 0.19
	0.1	0.78 ± 0.06**	1.03 ± 0.02	1.17 ± 0.21
	1	0.78 ± 0.04**	0.98 ± 0.05	1.02 ± 0.14
	PC	1.09 ± 0.11	4,57 ± 0.79***	11.07 ± 4.71***
GADD45α	0	1.00 ± 0.05	1.00 ± 0.01	1.00 ± 0.02
	0.01	0.83 ± 0.04***	1.21 ± 0.08**	1.39 ± 0.24**
	0.1	0.81 ± 0.12**	1.21 ± 0.12*	1.41 ± 0.20***
	1	0.84 ± 0.02**	1.27 ± 0.20*	1.25 ± 0.20*
	PC	1.04 ± 0,15	3.20 ± 0,90**	6.44 ± 1.44***
MDM2	0	1.00 ± 0.08	1.00 ± 0.02	1.00 ± 0.10
	0.01	0.91 ± 0.06	1.01 ± 0.15	1.13 ± 0.12
	0.1	0.86 ± 0.10	1.03 ± 0.08	1.21 ± 0.00*
	1	0.88 ± 0.04	0.92 ± 0.04	0.94 ± 0.19
	PC	0.72 ± 0.23*	0.70 ± 0.11**	0.79 ± 0,07**
BAX	0	1.00 ± 0.04	1.00 ± 0.05	1.00 ± 0.06
	0.01	0.94 ± 0.01	1.03 ± 0.07	1.11 ± 0.04
	0.1	0.92 ± 0.10	0.99 ± 0.06	1.11 ± 0.07
	1	0.93 ± 0.08	0.96 ± 0.04	0.97 ± 0.03
	PC	0.99 ± 0.13	0.99 ± 0.13	1.35 ± 0.10***
BCL2	0	1.00 ± 0.13	1.00 ± 0.02	1.01 ± 0.16
	0.01	0.80 ± 0.14	0.87 ± 0.24	1.50 ± 0.41
	0.1	0.86 ± 0.07	0.91 ± 0.22	1.61 ± 0.37*
	1	0.91 ± 0.13	1.00 ± 0.26	1.43 ± 0.29*
	PC	1.05 ± 0.30	0.08 ± 0.06***	0.03 ± 0.01***

The asterisks denote significant differences between NOD-treated groups and the vehicle control (0) (Student's t-test; (*) $p<0.05$, (**) $p<0.01$ and (***) $p<0.001$). PC is the positive control group (BaP 30µM).

Exposure to the tested concentrations of NOD did not induce any changes in the mRNA expression of *P53* (Table 1). It caused slight though significant down-regulation of *CDKN1A* and *GADD45α* after 4 h of exposure. However, after 12 and 24 h it induced significant dose and time dependant up-regulation of *GADD45α* at al concentrations tested. There was only slight up regulation of *MDM2* after 24 h of exposure to 0.1 µg/ml NOD. The anti-apoptotic gene *BCL2* was significantly up-regulated after 24 h of exposure to 0.1 and 1 µg/ml NOD, while there were no significant changest in the expression of the pro-apoptotic gene *BAX*.

4 Discussion

In this study we describe genotoxic effects of NOD and its influence on the induction of oxidative stress in HepG2 cells by measuring formation of reactive oxygen species (ROS) and oxidative DNA damage, and transcriptional changes in genes involved in the DNA damage response.

NOD was not cytotoxic to HepG2 cells at the tested conditions and caused only slight DNA strand breaks after 12 and 24 h of exposure. This is in agreement with the results of Lankoff et al. (2006) (Lankoff et al. 2006), who reported positive comet assay results in HepG2 cells only at higher NOD concentrations (5 µg /ml and 2.5 µg /ml after 6 and 12 h of exposure, respectively).

To explore whether NOD causes increased formation of ROS in HepG2 cells the DCFH-DA probe was applied. The results showed dose and time dependent increase of intracellular ROS. Our results are consistent with other publications, reporting that NOD induces oxidative stress (Lankoff, Banasik et al. 2002) and produces increased intracellular ROS, which is associated with modulation of intracellular glutathione content and lipid peroxidation (Bouaïcha and Maatouk 2004). NOD (2 and 10 ng/ml) was also previously reported to induce time and dose dependent formation of 8-oxo-dG adducts in primary cultured rat hepatocytes (Maatouk, Bouaďcha et al. 2004), and induction of oxidative DNA damage by NOD was already shown in HepG2 cells using the modified comet assay with the Fpg enzyme, however higher concentrations than the ones used in our study were applied (1, 2.5, 5 and 10 µg/ml) (Lankoff, Wojcik et al. 2006). The results of our study show that the highest level of oxidative DNA damage was reached already after 4 h of exposure to NOD, inducing significant damage already at the lowest tested concentration (0.01 µg/ml). The level of oxidative DNA damage remained high also after 12 and 24 h at higher NOD concentrations. This is in contrast to the study by Lankoff et al (2006) (Lankoff, Wojcik et al. 2006) who reported the highest level oxidized purines after 24 h exposure. In both studies DNA strand breaks level (whidhout enzymatic digestion) peaked at 12 h of exposure.

In the present study changes in the expression of *P53* and its downstream target genes (*CDKN1A*, *GADD45α*, *MDM2*, *BAX* and *BCL2*) were measured, at the same NOD concentrations and time points as DNA damage. Toxicogenomic analysis are becoming widely used as a tool for hazard identification and risk assessment of genotoxic and carcinogenic properties of different substances, as an addition to conventional genotoxicity assays (Ellinger-Ziegelbauer et al. 2009). Changes in the expression of *P53* and the P53-regulated DNA-damage response genes, like *CDKN1A*, *MDM2* and *GADD45A*, can be considered as markers of genotoxic and carcinogenic stress (Vogelstein et al. 2000).

NOD induced only minor changes in the expression of the selected genes involved in the DNA damage response and apoptosis. We could not detect any change in the expression of *P53* or *BAX* at any tested concentration and time of exposure to NOD. After 4 h of exposure down-regulation of *CDKN1A* and *GADD45α* was observed, while after 12 and 24 h NOD up-regulated *GADD45α* in a dose dependant manner. The tumour-suppressor gene, *P53*, plays the central role in the cellular response to agents or conditions that damage DNA by activating the transcription of several essential genes controlling cell cycle arrest, DNA repair, senescence, differentiation and apoptosis (Vogelstein et al. 2000). The concentration of P53 in the cell is regulated mainly through a negative feedback loop. Elevated levels of P53 up-regulate the expression of *MDM2*, which encodes MDM2, an ubiquitine-ligase, that mediates ubiquitination of P53, targeting it for proteosomal degradation (Michael and Oren 2002; Vogelstein et al. 2000). *MDM2* was significantly up-regulated only after 24 h of exposure to 0.1 µg/ml of NOD. The main target of P53 upon DNA damage is *CDKN1A*, that encodes $P21^{WAF1/CIP1}$, an inhibitor of cyclin-dependent kinases (CDKs), that inhibits the cell-cycle at the G1-S and the G2-M transitions (Michael and Oren 2002; Vogelstein et al. 2000). The observed changes in the expressions of DNA damage responsive genes, could not confirm induction of the P53 network by NOD, as there was no induction of *P53* and only slight changes in the expression of its target genes were detected. However its induction cannot be rouled out as only a limited number of its target genes were analysed. In addition we could have missed the timepoint of the induction of the analysed genes.

The *GADD45A* gene can be induced through P53-dependent and -independent pathways (Smith et al. 1994). Its product plays important role in the control of cell cycle G2-M checkpoint (Jin et al. 2000; Wang et al. 1999), induction of cell death (Harkin et al. 1999; Takekawa and Saito 1998) and DNA repair process (Hollander et al. 2001; Smith et al. 1994; Smith et al. 1996). Its up-regulation after NOD exposure indicates that NOD causes genotoxic stress in HepG2 cells, as is known that *GADD45* genes are induced rapidly by a wide spectrum of genotoxic agents (Dickinson et al., 2004; Ellinger-Ziegelbauer et al., 2005; Heise et al., 2012).

Oxidative stress is closely related to apoptotic cell death and was shown to induce apoptosis in several cell types (for review see (Slater et al., 1995; Dröge, 2002)). The lack of induction of the pro-apoptotic gene *BAX* and the up-regulation of the anti-apoptotic gene *BCL2* however, indicates supression of apoptosis as the ratio of death antagonists to agonists determines whether a cell will respond to an apoptotic signal (Kroemer, 1997).

5 Conclusions

Based on the results of the present study, we can conclude, that oxidative stress plays a substantial role in the genotoxicity of NOD in HepG2 cells as the toxin induced increased ROS production and oxidative DNA damage already at low environmentaly relevant concentrations.

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Declaration of interest:
The authors report no conflict of interest.

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2.2.4 Testiranje presnavljanja CYN

Princip metod testiranja mikrosomalne stabilnosti in stabilnost v prisotnosti primarnih humanih hepatocitov

Kar se tiče presnove (metabolizma) ksenobiotikov, so jetra najpomembnejši organ v človeškem telesu. Izolirani humani hepatociti so dober *in vitro* model za predvidevanje jetrne presnove preiskovane snovi *in vivo*, saj vsebujejo popolni komplet tako encimov I. kot II. faze, ki so prisotni v intaktni celici in omogočajo predvidevanje presnove v prisotnosti humanih jetrnih metaboličnih encimov in določanje intrinzičnega očistka *in vitro*. Intrinzični očistek učinkovine odraža sposobnost jeter za metabolično odstranjevanje nevezane učinkovine.

Tudi subcelične frakcije, kot so jetrni mikrosomi, predstavljajo koristne *in vitro* modele jetrnega metabolizma, saj vsebujejo večino jetrih metaboličnih encimov I. faze. Približno 60 % vseh komercialnih spojin se presnavlja s hepatičnimi encimi I. faze, citokromi iz družine P450 (CYP450). Test mikrosomalne stabilnost se uporablja kot presejalni test za odkrivanje raznih potencialnih farmacevtikov *in vitro*. Omogoča določitev, ali se snov presnavlja v prisotnosti humanih jetrnih metaboličnih encimov I. faze in določanje intrinzičnega očistka *in vitro*. Za zmanjšanje učinka interindividualne variabilnosti je posamezna testna mikrosomalna frakcija sestavljena iz frakcij različnih darovalcev hepatocitov.

Postopek

Test so opravili v podjetju Cyprotex, ki zagotavlja ponovljivost, visoko kakovost podatkov in visoko avtomatiziranost postopka.

Primarne humane hepatocite in mikrosome so inkubirali s CYN pri 37°C. Mikrosomalno reakcijo so sprožili z dodatkom kofaktorja, NADPH. Po koncu poizkusa so reakcijo ustavili z dodatkom metanola. Po centrifugiranju so supernatant analizirali s tekočinsko kromatografijo s tandemsko masno spektrometrijo (LC-MS/MS). Presnavljenje CYN so spremljali 45 minut v primeru testa mikrosomalne stabilnosti in 60 min v primeru testa stabilnosti v prisotnosti hepatocitov. Intrinzični očistek so izračunali po formuli (1) in razpolovni čas ($t_{1/2}$) po formuli (2), pri čemer je k eliminacijska konstanta, ki jo predstavlja formula (3), V pa so izračunali po formulah (4) za test mikrosomalne stabilnosti in (5) za test stabilnosti v prisotnosti hepatocitov.

$$CLint = V \times k \quad \ldots(1)$$

$$t\frac{1}{2}(min) = 0{,}693/k \quad \ldots(2)$$

$$k = -naklon\ krivulje\ upadanja \quad \ldots(3)$$

$$V(\mu l/mg) = \frac{inkubacijski\ volumen\ (\mu l)}{proteini\ (mg)} \quad \ldots(4)$$

$$V(\mu l/mg) = \frac{inkubacijski\ volumen\ (\mu l)}{število\ celic} \quad \ldots(5)$$

Rezultati

V prisotnosti primarnih humanih hepatocitov se CYN ni presnavljal. Intrinzični očistek je bil zelo nizek (1,19 µl/min/10^6 celic) in razpolovni čas so izračunali pri 1160 min, kar kaže, da se CYN pri teh testnih pogojih ne presnavlja (Tabela 1). V prisotnosti mikrosomov je CYN imel srednje visok intrinzični očistek (16,9 µl/min/mg) in razpolovni čas pri 82 min (Tabela 2), kar kaže na to, da se CYN presnavlja z metaboličnimi encimi I. faze.

Pregl. 1: Testiranje stabilnosti CYN v prisotnosti primarnih humanih

Tab. 1: Stability of CYN in the presence of human primary hepatocytes

		Intrinzični očistek (µL/min/10^6 celic)	**$t_{1/2}$ (min)**	**n**
CYN		1.19	1160	6
Kategorija očistka	nizek	< 3.5	Tipične vrednosti za klasifikacijo snovi v skupine z nizkim, srednjim in visokim očistkom.	
	Visok	> 19.0		

Pregl. 2: Testiranje mikrosomalne stabilnosti CYN

Tab. 2: Microsomal stability of CYN

		Intrinzični očistek (µL/min/mg)	**$t_{1/2}$ (min)**	**n**
CYN		16.9	82.0	6
Kategorija očistka	nizek	< 8.6	Tipične vrednosti za klasifikacijo snovi v skupine z nizkim, srednjim in visokim očistkom.	
	Visok	> 47.0		

2.2.5 Določanje mutagenosti MCLR, NOD in CYN s testom AMES

Princip metode

Amesov test (ali *Salmonella* mikrosomalni test povratnih mutacij) je široko uporabljena metoda za določanje mutagenov (Maron in Ames, 1983). Pri tem testu se uporabljajo različni od histidina odvisni sevi bakterije *Salmonella typhimurium* (*S. typhimurium*) ali od triptofana odvisni sevi bakterije *Escherichia coli*. V doktorski disertaciji smo za testiranje mutagenosti MCLR, NOD in CYN uporabili bakterije *S. typhimurium*. Te bakterije so histidinski avksotrofi (His-) zaradi mutacij v različnih genih na histidinskem operonu in ne morejo sintetizirati aminokisline histidin ter posledično ne morejo rasti v njegovi odsotnosti. Nove mutacije na mestih teh predhodnih mutacij, ali v njihovi bližini, lahko ponovno vzpostavijo divji tip bakterij – prototrof (His+) –, ki lahko tvori kolonije v odsotnosti dodanega histidina. Število spontanih revertant na ploščo je relativno konstantno. V prisotnosti mutagenov se število povratnih mutacij poveča. Tem bolj je neka snov mutagena, tem več mutacij nastane, kar posledično zaznamo s povečanim številom kolonij na ploščah.

Sevi bakterije *Salmonella typhimurium*, ki jih uporabljamo v Amesovem testu, so pridobljeni iz bakterije *Salmonella enterica* subsp. I serovar *Typhimurium* (*S. typhimurium* LT2) in imajo poleg mutacij v različnih genih na histidinskem operonu (his-) vnešene dodatne mutacije za povečanje njihove občutljivosti. Za določanje mutagenosti izbranih cianobakterijskih toksinov smo uporabili seva TA100 (*hisG46*, *rfa*-, Δ*uvrB*, R+) in TA98 (*hisD3052*, *rfa*-, Δ*uvrB*, R+).

Kazalec *hisG46* seva TA100 se uporablja za ugotavljanje mutacij, kjer pride do zamenjave baznih parov, primarno na GC baznih parih (zamenjava leucina (GAG/CTC) s prolinom (GGG/CCC)). Kazalec *hisD3052* vstavljen v sev TA98 pa zaznava mutacije, kjer pride do delecije baznega para in posledično -1 premika bralnega okvirja blizu ponavljajoče sekvence -C-G-C-G-C-G-C-G-. Dodatno imata seva delecijo *uvrB*, mutacijo *rfa* in vsebujeta plazmid pKM101. Zaradi delecije *uvrB* bakterije ne morejo popravljati DNK poškodb z DNK izrezovalnim popravljanjem, temveč preko popravljanja z napakami (ang.: error-prone), kar poviša stopnjo kemične mutageneze in mutageneze povzročene z UV-svetlobo. Delecija *uvrB* se razteza preko gena za sintezo biotina, zaradi česar so bakterije odvisne tudi od aminokisline biotin. Mutacija *rfa* poveča propustnost lipopolisaharidnega ovoja, ki pokriva površino bakterije in je tako bolj prepusten za prehajanje večjih molekul. Plazmid pKM101 nosi *muc* gene, ki prav tako povišajo frekvenco popravljanja DNK poškodb z »error-prone« popravljalnim mehanizmom. Vsebuje tudi gen *bla*, ki kodira β-laktamazo in tako posreduje rezistenco na ampicilin (Amp^r), *Rep* regijo, ki omogoča replikacijo plazmida, *Slo* regijo, ki je odgovorna za zmanjšanje celične rasti na minimalnem mediju, *tra* gene, ki omogočajo konjugacijo ter enkratni restrikcijski mesti za EcoRI in BamHI.

Postopek

Standardni test vgradnje v ploščo

Pri standardnem testu z vključitvijo na ploščo izpostavimo testni sev kemikaliji direktno na plošči minimalnega glukoznega gojišča (MGG).

Seva TA100 in TA98 smo dan pred testom inokulirali v hranilni bujon (2,5 % Nutrient Broth N°2 Oxoid, 25 μg/ml ampicilin) in pustili čez noč (največ 16 h) na 37°C. Naslednji dan smo kulturo stresali (≈ 600 rpm, 1,5 - 2 h), tako da so bakterije prešle v eksponentno fazo rasti. Epruvete smo segreli na ≈ 43°C in vanje dodali 2 ml mehkega površinskega agarja (0,6 % agar, 0,6 % Nacl, 50 μM histidin/biotin). Pazili smo, da temperatura ni presegla 45°C in da bakterije niso bile predolgo na visoki temperaturi. Pripravili smo rečitve izbranih cianobakterijskih toksinov, tako da smo imeli na plošči končne koncentracije 0,01, 0,1 in 1 μg/ploščo MCLR in NOD ter 0,005, 0,05 in 0,5 μg/ploščo CYN. V epruvete smo dodali 100 μl ustrezne rečitve cianobakterijskih toksinov, 100 μl suspenzije bakterij in 500 μl fosfatnega pufra. V primeru CYN smo napravili test z vgradnjo v ploščo tudi z metabolično aktivacijo, kjer smo namesto fosfatnega pufra dodali 500 μl S9 mešanice [4 % S9 – mikrosomalna frakcija podganjih jeter (Moltox, Boone, NC, USA)]. Zmes smo premešali in razlili na plošče MGG. Kot pozitivno kontrolo smo uporabili 4-nitrokinolin-N-oksid (4-NQNO, 0,5 μg/ploščo) pri testiranju brez aktivacije in benzo[a]piren (BaP, 10 μg/ploščo) pri testiranju z aktivacijo. Plošče smo inkubirali pri 37°C za 2 (TA100) oziroma 3 (TA98) dni. Po končani inkubaciji smo prešteli kolonije revertant. Za vsako testno točko smo pripravili po tri paralelne plošče. Rezultate smo izrazili kot srednjo vrednost števila zraslih kolonij na treh ploščah ± SD. Za oceno mutagenosti smo upoštevali dva kriterija: od doze odvisno naraščanje števila induciranih revertant in podvojitev števila induciranihrevertant glede na število spontanih revertant pri vsaj eni koncentraciji.

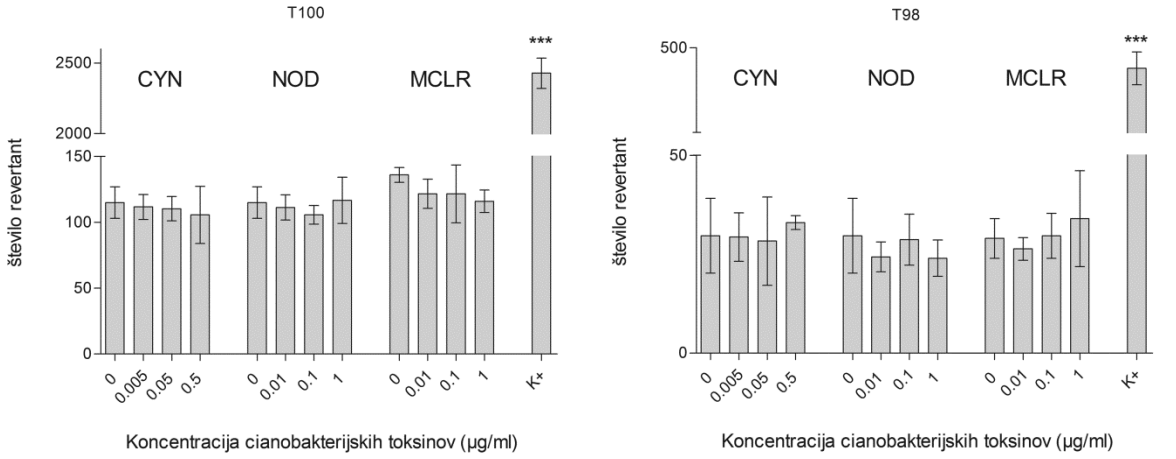
Test s predinkubacijo

Test s predinkubacijo je različica standardnega testa vgradnje v ploščo, vendar je optimiziran za kratkoživeče mutagene metabolite, ki tako lahko bolje reagirajo z bakterijsko kulturo v manjšem volumnu predinkubacijske mešanice z višjimi efektivnimi koncentracijami S9 in kofaktorjev.

Test smo izvedli enako kot standardni test vgradnje v ploščo, vendar smo pred vgradnjo v plošče bakterije (100 μl) inkubirali 30 minut na 37°C skupaj s 100 μl pripravljenih razrečitev CYN in 500 μl 4 % S9 mešanice.

Rezultati

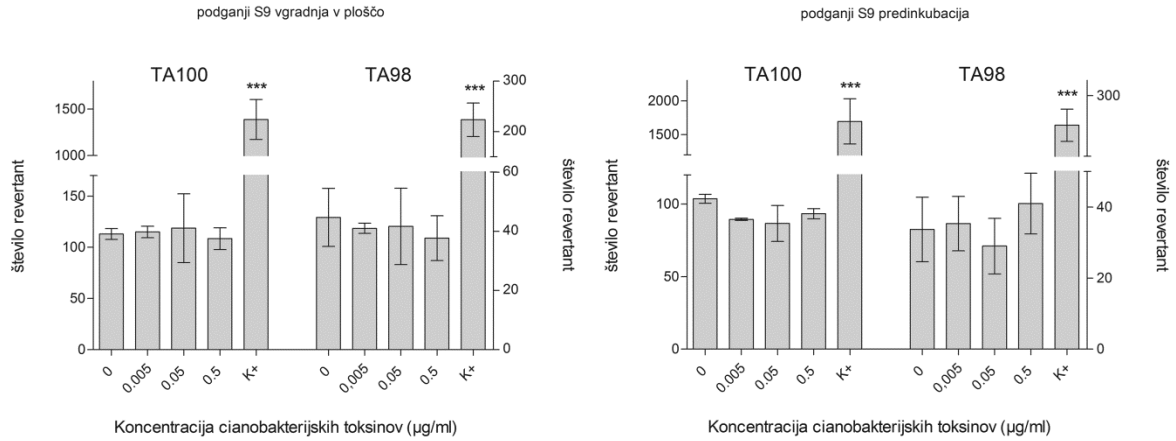
Pri pogojih testiranja nobeden izmed testiranih cianotoksinov (CYN, NOD in MCLR) ni povišal števila revertantov (F>2) (Slika 1), zato jih obravnavamo kot nemutagene v bakterijskem testu povratnih mutacij. Metabolična aktivacija s testom z vgradnjo v ploščo prav tako kot s predinkubacijo ni vplivala na število revertant induciranih s CYN (Slika 2).



Sl. 1: Testiranje mutagenosti CYN, NOD in MCLR

Fig. 1: Analisys of the mutagenic potential of CYN, NOD and MCLR

Testiranje mutagenosti CYN, NOD in MCLR s sevoma *Salmonella typhimurium* TA100 (levo) in TA98 (desno) s standardnim testom Ames vgradnje v ploščo brez metabolične aktivacije. K+ – pozitivna kontrola: 4-NQNO (0,5 µg/ploščo).



Sl. 2: Testiranje mutagenosti CYN z metabolično aktivacijo

Fig. 2: Analisys of the mutagenic potential of CYN using metabolic activation

Testiranje mutagenosti CYN s sevoma *Salmonella typhimurium* TA98 in TA100 s standardnim testom Ames vgradnje v ploščo (levo) in s predinkubacijo (desno). K+ – pozitivna kontrola: BaP (10 µg/ploščo).

3 RAZPRAVA IN SKLEPI

3.1 RAZPRAVA

3.1.1 Genotoksična aktivnost CYN, MCLR in NOD

Cilindrospermopsin – CYN

Analiza poškodb DNK s testom komet, s katerim lahko zaznamo eno- in dvoverižne prelome DNK in alkalno labilna mesta, je pokazala, da je CYN genotoksičen za celice HepG2 kot tudi za primarne humane limfocite (ang.: human peripheral blood lymphocytes, HPBL). CYN je pri celicah HepG2 povzročil prelome DNK po izpostavitvi celic necitotoksičnim koncentracijam za 12 h, le pri najvišji testirani koncentraciji (0,5 µg/ml), in za 24 h že pri 50-krat nižji (0,01, 0,05, 0,1 in 0,5 µg/ml). Pri HPBL je CYN povzročil prelome DNK že po 4 h izpostavitve najvišji koncentraciji (0,5 µg/ml). Po 24 h je bila količina prelomov DNK statistično značilno povišana pri koncentracijah 0,05 in 0,1 µg/ml. Ti rezultati se skladajo z objavama, ki sta pokazali poškodbe DNK pri primarni mišjih hepatocitih *in vitro* (Humpage s sod., 2005) in v jetrnih celicah miši *in vivo* (Shen s sod., 2002).

CYN je povzročil tudi od koncentracije odvisno povišanje nastajanja mikrojeder (MNi), jedrnih brstov (NBUD) in nukleoplazmatskih mostičkov (NPB) pri HepG2 celicah ter povišano nastajanje MNi in NBUD pri HPBL. Test mikrojeder je najpogosteje uporabljena metoda za zaznavanje nastajanja MNi, ki so posledica prelomov ali izgube celih kromosomov med delitvijo celice. S tem testom lahko zaznamo tudi druge genomske nepravilnosti, kot so NBUD, ki so kazatelji genetskih preamplifikacij, in NPB, ki so posledica napak pri popravljanju DNK, kromosomskih premestitev in fuzij telomer (Fenech, 2006). Pri celicah HepG2 smo zaznali statistično značilno povišanje MNi, celic z MNi in NBUD po izpostavitvi CYN za 24 h pri koncentracijah 0,05 in 0,5 µg/ml. Število MNi pri najvišji koncentraciji je bilo več kot 3-krat višje kot pri kontroli, število NBUD pa 2,6-krat. Rahlo se je povišalo tudi število NPB. CYN je povzročil povišanje števila MNi in povišal frekvenco celic z MNi tudi pri HPBL že pri 4 h izpostavitve koncentraciji 0,5 µg/ml. Po 24 h smo zaznali od koncentracije odvisno povišanje, ki je bilo statistično značilno že pri 5-krat nižji koncentraciji (0,1 in 0,5 µg/ml). Število NBUD je bilo prav tako povišano po 4 (0,05 in 0,1 µg/ml) in 24 h (0,1 µg/ml). Povišana frekvenca MNi po izpostavitvi CYN sovpada z drugimi študijami, ki so pri primerljivih koncentracijah pokazali povišano nastajanje MNi pri limfoblastoidni celični liniji WIL2-NS (Humpage s sod., 2000), kjer so pokazali klastogeno in aneugeno delovanje CYN, CaCo-2 celicah črevesnega izvora in diferenciranih jetrnih celicah HepaRG (Bazin s sod., 2010).

Glede na rezultate testa mikrojeder v naših in v drugih študijah (Shen s sod., 2002; Humpage s sod., 2005; Bazin s sod., 2010) je pričakovano, da CYN povzroča dvoverižne prelome DNK (DSB). DSB so za celico najbolj škodljiva oblika DNK poškodb, saj lahko vodijo v prelome kromosomov in kromosomske premestitve (Dasika s sod., 1999). Takšne poškodbe v celici aktivirajo različne celične procese, kot so signalizacijske poti AP-1 in NF-kB, aktivacija kontrolnih točk celičnega cikla, popravljanje poškodb preko HRR ali NHEJ in sprožitev apoptoze preko P53 (Norbury in Hickson, 2001; Habraken in Piette, 2006). Nastanku DSB sledi fosforilacija histona H2AX, ki je sestavni del histonskega octomera v nukleosomih (Rogakou s sod., 1998; Sedelnikova s sod., 2002). Fosforilirni histoni H2AX (γH2AX) se kopičijo na mestih DSB. Fiziološka vloga tega pojava še ni poznana, domneva pa se, da povzroči razrahlanje kromatina na mestih DSB in tako omogoči dostop popravljalnim encimom (ref). Predpostavlja se, da žarrišča γH2AX v celicah predstavljajo DSB v razmerju 1: 1 (Rogakou s sod., 1999; Sedelnikova s sod., 2002) in se tako uporabljajo kot kazalci za DSB. V doktorski disertaciji smo prisotnost DSB pri celicah HepG2, izpostavljenih CYN, analizirali s pretočno citometrijo, preko označevanja γH2AX z monoklonskimi flurescentno označenimi protitelesi. Po 24 h izpostavitve nastanka DSB nismo zaznali, medtem ko je po daljši izpostavljenosti (72 h) CYN povzročil DSBs pri najvišji preskušani koncentraciji (0,5 µg/ml).

V obeh testnih sistemih (celicah HepG2 in HPBL) je CYN povzročil tudi spremembe v izražanju genov, vpletenih v odgovor na poškodbe DNK. Tako imenovane toksikogenomske analize se vse pogosteje uporabljajo za določanje genotoksičnih in karcinogenih snovi, dodatno k standardni bateriji testov genotoksičnosti (Ellinger-Ziegelbauer s sod., 2009). Pogosto se kot kazalci genotoksičnega stresa preučujejo spremembe v izražanju gena *P53* in z njim regulirani geni, kot so *CDKN1A, GADD45a in MDM2*, ki so vpleteni v odgovor na poškodbe DNK, kot so ustavitev celičnega cikla, popravljanje DNK, senescenca in apoptoza (Vogelstein s sod., 2000). *P53* je tumor supresorski gen, ki kodira transkripcijski faktor, ki igra centralno vlogo pri celičnem odzivu na agense in razmere, ki poškodujejo DNK. Skoraj v vseh sesalčjih celicah je ustavitev celičnega cikla prvi odziv na poškodbe DNK. *CDKN1A*, kodira inhibitor od ciklina odvisnih kinaz (CDKI), ki zavira celični cikel v G1 in G2 fazah (Vogelstein s sod., 2000). Proteinski produkt gena *GADD45α* prav tako vpliva na ustavitev celičnega cikla v G2 fazi, sprožitev popravljanja DNK poškodb in apoptoze (Smith s sod., 1994; Zhan s sod., 1999; Zhan, 2005). *MDM2* pa kodira onkogen, ki preko negativne povratne zanke nadzira količino P53 proteina v celici (Wu s sod., 1993; Momand s sod., 2000).

Pri HPBL po izpostavitvi CYN za 24 h smo zaznali statistično značilno povišanje izražanja gena *P53*. V nasprotju pri celicah HepG2 sprememb v izražanju *P53* nismo zaznali, kar ni nenavadno, saj je količina aktivnega proteina P53 v večji meri odvisna od fosforilacije obstoječega proteina v citoplazmi preko kinaz, ki se odzivajo na poškodbe DNK, in v manjši meri od povišanega izražanja gena (Zhou in Elledge, 2000). Poleg tega je izražanje

lahko povišano le kratek čas, ki pa smo ga lahko zgrešili. Rezultati sovpadajo s študijo na celicah HepG2, kjer niso zaznali sprememb v izražanju gena, vendar so zaznali kopičenje proteina P53 po 48 h izpostavitve CYN (Bain s sod., 2007). V isti študiji so pokazali tudi, da CYN vpliva na izražanje *CDKN1A, GADD*45α, *MDM*2 in *BAX* pri celicah HepG2 in humanih kožnih fibroblastih (HDF). Koncentracije CYN, ki so jih uporabili so bile mnogo višje (1−5 µg/ml) od koncentracij, uporabljenih v naši študiji, kjer smo jih že zaznali kot citotoksične. Povišanje izražanja genov *CDKN1A, GADD45a in MDM2* v naši študiji na celicah HepG2 je sovpadalo z nastankom poškodb DNK. Tako po 4 h izpostavitve nismo zaznali sprememb v izražanju. Pri najvišji koncentraciji CYN (0,5 µg/ml) smo po 12 h izpostavitvi zaznali statistično značilne spremembe v izražanju genov *GADD45α* in *MDM2*, po 24 h pa je bilo dodatno značilno povišano tudi izražanje *CDKN1A.* Tudi pri HPBL je CYN statistično značilno povišal izražanje genov *GADD45α* in *MDM2* po 24. h izpostavitvi, medtem ko sprememb v izražanju *CDKN1A* nismo zaznali.

Glavni tarčni organ CYN so jetra, prizadene pa lahko tudi druge organe (ledvice, pljuča, priželjc, vranico, nadledvično žlezo, prebavni trakt, imunski sistem in srce) (Hawkins s sod., 1985; Terao s sod., 1994; Falconer s sod., 1999; Humpage s sod., 2000). Kljub temu da limfociti niso znana tarča delovanja CYN, naši rezultati kažejo, da so tarča genotoksičnega delovanja CYN, čeprav je le-ta povzročil manj poškodb pri HPBL kot pa pri celicah HepG2. Razlog za to bi lahko bila manjša metabolična aktivnost HPBL. Drugi razlog bi lahko iskali v načinu izpostavitve celic, saj smo v primeru raziskave delovanja na HPBL toksinu izpostavili polno kri, kjer so bile prisotne tudi druge krvne celice in proteini plazme ter antioksidanti (GSH), zaradi česar so bili limfociti morebiti bolj zaščiteni pred delovanjem CYN.

Domneva se, da se CYN aktivira z encimi iz družine CYP450 (Norris s sod., 2002; Humpage s sod., 2005; Bazin s sod., 2010; Štraser s sod., 2011; Žegura s sod., 2011) in ker so reakcije, ki jih katalizirajo ti encimi, pomemben endogeni vir nastajanja reaktivnih kisikovih zvrsti (ROS) (Parke, 1994; Olinski s sod., 2002; Klaunig in Kamendulis, 2004), bi CYN lahko povzročal oksidativni stres in s tem oksidativne poškodbe DNK. Poleg tega naj bi zaviral tudi sintezo glutationa (Runnegar s sod., 1995; Humpage s sod., 2005), ki igra centralno vlogo v preprečevanju oksidativnega stresa v celici. Vendar pa so si študije o vplivu CYN na oksidativni stres nasprotujoče. Dve novejši študiji na celicah HUVEC (Gutiérrez-Praena s sod., 2012) in CaCo-2 (Gutiérrez-Praena s sod., 2012) sta pokazali, da CYN poviša raven glutationa v celici in aktivnost encima γ-glutamilcistein sintetaze, ki je vpleten v njegovo sintezo, najverjetneje kot odgovor na povečano nastajanje ROS, ki so ga zaznali po izpostavitvi celic CYN. V nasprotju je Humpage s sod. (2005) pokazal, da izpostavitev mišjih hepatocitov CYN ne povzroča lipidne peroksidacije, enega od kazalcev oksidativnega stresa (Humpage s sod., 2005).

Analiza tvorbe ROS s sondo DCFH-DA na celicah HepG2 je pokazala od koncentracije in časa odvisen nastanek ROS po izpostavitvi celic CYN. Vendar pa kljub povečanemu nastanku ROS, zaradi delovanja CYN, z modificiranim testom komet nismo zaznali oksidativnih poškodb DNK po 4 h izpostavitve. Pri testu smo uporabili encim formamidopirimidin glikozilazo (Fpg), ki prepozna in izrezuje oksidirane purine (Collins s sod., 1993). Pri vzporednem poizkusu brez encima smo po 12 in 24 h, podobno kot v prejšnji raziskavi (Štraser s sod., 2011), zaznali statistično značilno povišanje poškodb DNK. V enaki meri smo DNK poškodbe zaznali tudi po razgradnji s Fpg. Ker je razgradnja s Fpg znatno dvignila nivo bazalnih poškodb, glede na obe kontroli ni bilo razlik med poškodbami brez in z razgradnjo s Fpg, kar kaže na to, da CYN pri celicah HepG2 ne povzroča oksidativnih DNK poškodb.

Rezultati transkriptomske študije s QPCR čipi na celicah HepG2 so pokazali spremembe v izražanju genov, ki so vpleteni v odgovor na oksidativni stres (Glej 3.1.2), vendar pa lahko na podlagi teh rezultatov ponovno sklepamo, da CYN ni povzročil znatnega oksidativnega stresa pri celicah HepG2. V nasprotju smo pri HPBL zaznali povišano izražanje večine testiranih genov (*GPX1*, *GSR*, *GCLC* in *SOD1*), vpletenih v odziv na oksidativni stres, po 24 h izpostavitvi CYN.

Mikrocistin-LR – MCLR

Najpogostejši predstavnik mikrocistinov (MCs) je mikrocistin-LR (MCLR), ki je izmed treh cianotoksinov, vključenih v doktorsko disertacijo, najbolje raziskan. V številnih študijah *in vitro* in *in vivo* so pokazali, da MCLR povzroča poškodbe DNK, ki so večinoma oksidativne narave in so posledica povišanega nastajanja ROS. Poleg tega poviša frekvenco MNi kot posledica izgube dela ali celega kromosoma (za pregled glej (Žegura s sod., 2011)). Pri HPBL MCLR ni povzročil povišanega nastanka MNi, NBUD ali NPB. Povzročil je od koncentracije odvisno povišanje poškodb DNK po 6 h (1 in 10 µg/ml) in 24 h (pri vseh testiranih koncentracijah: 0,1, 1 in 10 µg/ml) izpostavitve necitotoksičnim koncentracijam. Poškodbe so bile, skladno z drugimi študijami, oksidativne narave, saj smo zaznali povišanje na Fpg občutljivih mest. Tudi oksidativne poškodbe smo zaznali šele po 6 h izpostavitve CYN, največ pa jih je bilo po 24 h. Podobne rezultate na HPBL izpostavljene MCLR so opisali tudi Lankoff s sod. (2004), vendar pri višjih koncentracijah (1, 10 in 25 µg/ml), zaradi česar bi opisane poškodbe DNK lahko pripisali citotoksičnosti MCLR (Lankoff s sod., 2004). Različne študije na HPBL so pokazale različno kinetiko nastajanja DNK poškodb, z viškom po 3 in 6 h izpostavitve (Palus s sod., 2007), 12 h (Mankiewicz s sod., 2002) ali 18 h (Lankoff s sod., 2004). Ti podatki se razlikujejo od rezultatov na celicah HepG2, kjer so bili prelomi DNK opazni po 4 h, in so bili posledica prehodnih prelomov, ki nastanejo med popravljanjem DNK poškodb (Žegura s sod., 2003; Žegura s sod., 2004). Razlike bi lahko bile posledica hitrejšega prehajanja MCLR v celicah

HepG2, ali manj učinkovitega popravljanja DNK poškodb pri HPBL, ki so v nasprotju z aktivno rastočimi celicami HepG2 v stacionarni fazi rasti.

V številnih študijah, kjer so raziskovali spremembe, ki jih povzroča MCLR na transkripcijskem nivoju (za pregled glej (Žegura s sod., 2011)), so pokazali, da toksin vpliva na izražanje številnih genov, ki so vpleteni v odziv celice na poškodbe DNK, in številnih proto-onkogenov iz genskih družin *JUN* in *FOS*. Po drugi strani pa do sedaj ni nobenih podatkov o spremembah izražanja genov pri limfocitih izpostavljenih MCLR, prav tako ni podatkov o spremembah izražanja genov vpletenih v oksidativni stres. Pri HPBL po 4 h izpostavitve MCLR sprememb v izražanju genov nismo zaznali. Po 24 h izpostavitvi MCLR (1 µg/ml) je bilo izražanje vseh testiranih genov, vpletenih v odziv na poškodbe DNK (*P53*, *CDKN1A*, *GADD45α MDM2* in *BAX*) in odziv na oksidativni stres (*CAT*, *SOD1*, *GCLC*, *GSR* in *GPX1*), statistično značilno povišano, kar dodatno kaže, da je v genotoksično delovanje MCLR vpleten oksidativni stres.

Literaturnih podatkov o genotoksičnem delovanju MCLR je ogromno, medtem ko je manj zananega o delovanju drugih cianobakterijskih toksinov. Tako je bil MCLR po kriterijih mednarodne agencije za raziskave raka (IARC) uvrščen v skupino možnih karcinogenih snovi za ljudi (IARC skupina 2B), medtem ko je zaradi pomanjkanja podatkov NOD uvrščen med snovi s sumom na karcinogene lastnosti (IARC skupina 3) (IARC, 2010).

Nodularin – NOD

NOD je po strukturi in osnovnemu mehanizmu delovanja zelo podoben MCLR. Tudi zanj je znano, da lahko poveča nastajanje ROS (Bouaïcha in Maatouk, 2004) in s tem oksidativne poškodbe DNK (Bouaïcha in Maatouk, 2004; Lankoff s sod., 2006), po drugi strani pa poviša frekvenco MNi *in vitro* (Fessard s sod., 2004; Lankoff s sod., 2006).

Analiza testa komet je pokazala, da NOD po 12 in 24 h izpostavitve povzroča poškodbe DNK le v manjši meri. Ti rezultati se ujemajo z rezultati študije Lankoff in sod. (2006) (Lankoff s sod., 2006), ki so pokazali poškodbe s testom komet pri celicah HepG2 šele pri višjih koncentracijah NOD (5 µg/ml in 2.5 µg/ml po 6 in 12 h izpostavitve). Skladno s tem je NOD povzročil le manjše spremembe v izražanju genov, ki se odzovejo na poškodbe DNK. Sprememb v izražanju *P53* in *BAX* nismo zaznali pri nobeni od testiranih koncentracij in časih izpostavitve. Po 4 h izpostavitve je prišlo do znižanja v izražanju genov *CDKN1A* in *GADD45α*. Izražanje slednjega je bilo po 12 in 24 h izpostavitve povišano v odvisnosti od koncentracije. Izražanje *MDM2* je bilo rahlo, vendar statistično značilno povišano po 24 h izpostavitvi 0,1 µg/ml NOD. Po 24 h izpostavitve je bilo povišano tudi izražanje antiapoptotskega gena *BCL2*, kar nakazuje na zaviranje apoptoze.

NOD je pri celicah HepG2 povzročal nastanek ROS, kar se sklada z objavljenimi podatki (Lankoff s sod., 2002; Bouaïcha in Maatouk, 2004). Poročali so tudi o nastanku oksidativnih DNK poškodb pri primarnih mišjih hepatocitih po izpostavitvi NOD (2 in 10 ng/ml) (Maatouk s sod., 2004). Tudi modificirani test komet z encimom Fpg je pokazal oksidativne poškodbe DNK pri celicah HepG2, vendar pri višjih koncentracijah (1 -10 µg/ml) (Lankoff s sod., 2006) kot smo jih uporabili v doktorski disertaciji. Tudi kinetika nastajanja oksidativnih poškodb se je razlikovala. Medtem ko so Lankoff in sod. (2006) opazili največ poškodb po 24 h izpostavitve, naši rezultati kažejo najvišjo raven oksidativnih poškodb že po 4 h, kjer smo zaznali statistično značilno povišanje že pri najnižji testirani koncentraciji (0,01 µg/ml). Količina prelomov DNK brez encimske razgradnje je bila pri obeh študijah najvišja po 12 h izpostavitve.

Mutagenost CYN, MCLR in NOD

V sklopu standardne baterije testov genotoksičnosti se pogosto uporablja test Ames (ali *Salmonella* mikrosomalni test povratnih mutacij), ki je široko uporabljena metoda za določanje mutagenov (Maron in Ames, 1983). Nobena od testiranih koncentracij CYN, NOD in MCLR pri opisanih pogojih testiranja pri sevih *S. typhimurium* TA98 in TA100 ni povzročila povečanja števila revertant. Rezultati za MCLR se skladajo z objavljenimi podatki, ki kažejo da čisti MCs niso bakterijski mutageni (za pregled glej (Žegura s sod., 2011)), medtem ko za NOD in CYN še ni bilo objavljene študije na bakterijskih testnih sistemih V primeru CYN, ki je domnevno progenotoksičen, smo test izvedli še z metabolično aktivacijo s podganjo jetrno frakcijo S9, ki pa ni imela vpliva na število s CYN induciranih revertant ne pri standardnem testu z vgradnjo v ploščo kot tudi ne pri testu s predinkubacijo. Rezultati kažejo, da noben od treh cianotoksinov ni bakterijski mutagen, vendar je v primeru CYN možno, da za njegovo aktivacijo S9 ne zadostuje, saj ne vsebuje celotnega spektra CYP450 encimov ter ne vsebuje encimov II. faze. Nekatere snovi se aktivirajo z encimi, ki so v S9 inaktivirani ali prisotni le v nizkih koncentracijah (Ku s sod., 2007). Prav tako zunajcelična presnova snovi s S9 lahko vpliva na prehajanje v celice.

3.1.2 Vpliv CYN na transkriptomske spremembe celic HepG2

Da bi dobili vpogled v celični odziv na izpostavitev CYN, smo izvedli analizo izražanja 192 izbranih genov, ki se odzivajo na genotoksičen stres in so vpleteni v takojšnji zgodnji odziv (ang.: immediate early response), signaliziranje, celični cikel, popravljanje poškodb DNK, apoptozo in celično preživetje ter detoksifikacijo ksenobiotikov. Rezultati so pokazali številne statistično značilno spremenjene gene tako po 12 (17 %) kot 24 h (30 %) izpostavitvi CYN. Najpoglavitnejše in statistično relevantne spremembe so opisane v nadaljevanju.

Geni zgodnjega odziva in geni vpleteni v celično signalizacijo

CYN je močno vplival na izražanje genov iz genskih družin *JUN* in *FOS*. Njihovo izražanje je povišal za več kot 4-krat. CYN je povišal izražanje *FOS, FOSB* in *JUNB* že po 12 h izpostavitvi. Po 24 h je bilo povišanje še višje in povišalo se je tudi izražanje gena *JUN*. Geni zgodnjega odziva se aktivirajo hitro, njihovo izražanje je neodvisno od *de novo* sinteze beljakovin (Vogelstein s sod., 2000). Kodirajo transkripcijske faktorje oz. so del transkripcijskega kompleksa, aktivacijskega proteina AP-1, in regulirajo odziv na stres, ki je lahko ustavitev celičnega cikla, popravljanje poškodb DNK ali sprožitev apoptoze (Welch in Chrylogelos, 2002). Znano je, da izražanje teh genov povišajo številni genotoksični agensi (Hollander in Fornace, 1989; Sherman s sod., 1990). Izražanje genov iz družin *JUN* in *FOS* povišata tudi MCLR in NOD (za pregled glej (Žegura s sod., 2011)).

Povišano je bilo tudi izražanje gena *NFKB1*, ki kodira podenoto proteinskega kompleksa, transkripcijskega faktorja NF-κB. Spremenjeno izražanje številnih transkripcijskih tarč tega transkripcijskega faktorja skupaj s povišanim izražanjem *NFKB1*, nakazuje vpletenost signalne poti NF-κB v odziv celic na izpostavljenost CYN. Tudi ta signalna pot se odzove na številne genotoksične agense, ki povzročajo DSB (Reelfs s sod., 2004; Habraken in Piette, 2006; Janssens in Tschopp, 2006). Da CYN povzroča DSB, nakazujejo rezultati analize kopičenja γH2A.X, testa mikrojeder in številne spremembe v izražanju genov, vpletenih v popravljanje dvoverižnih poškodb.

Skladno z rezultati klasičnega kvantitativnega PCR v realnem času (QPCR) pri celicah HepG2 nismo zaznali sprememb v izražanju *P53*, vendar je spremenjeno izražanje njegovih številnih tarč kazalo na aktivacijo poti P53.

Geni vpleteni v regulacijo celičnega cikla

Transkripcijske spremembe genov vpletenih v regulacijo celičnega cikla in celične proliferacije so kazale na to, da CYN sproži ustavitev celičnega cikla.

CYN je povišal izražanje gena *CHEK1*, ki kodira kinazo kontrolne točke celičnega cikla in se aktivira v odgovor na poškodbe DNK ter ustavlja celični cikel na prehodih iz G1 v G2 in iz G2 v M fazo (Malumbres in Barbacid, 2009). CYN je spremenil tudi izražanje genov (*HUS1* in *RAD1*), ki kodirajo komponente kompleksa 9-1-1 (ang.: 9-1-1 cell-cycle checkpoint response complex), ki se odzove na poškodbe DNK tako, da se veže na mesto poškodbe, aktivira protein CHEK1 in tako koordinira aktivacijo kontrolnih točk celičnega cikla in popravljanje poškodb DNK (Parrilla-Castellar s sod., 2004).

Tudi produkt gena *CDKN1A*, $P21^{WAF1/CIP1}$, inhibitor od ciklina odvisnih kinaz (CDKI), ustavlja celični cikel na prehodih faz G1/G2 in G2/M (Vogelstein s sod., 2000; Michael in

Oren, 2002). Povišano izražanje tega gena se sklada z QPCR pri celicah HepG2 in rezultati, ki so jih objavili Bain s sod. (2007) (Bain s sod., 2007). V nasprotju pri HPBL povišanja izražanja tega gena nismo zaznali. CYN je spremenil tudi izražanje genov dveh drugih CDKI. Povišal je izražanje *CDKN2B* po 12 h izpostavitve in znižal izražanje *CDKN2C* po 24 h izpostavitve. Ta dva gena kodirata proteina $P15^{INK4B}$ in $P18^{INK4C}$, ki se vežeta in zavirata delovanje dveh od ciklina odvisnih kinaz (CDK) CDK4 in CDK6 (Serrano s sod., 1993) ter preprečujeta prehod iz G1 v S fazo celičnega cikla.

Zaznali smo tudi močno, od koncentracije in časa odvisno, povišanje izražanja z DNK poškodbami inducibilnih genov *GADD45α* in *GADD45β*. Povišano izražanje *GADD45α* se sklada z rezultati QPCR pri celicah HepG2 in HPBL in je bilo objavljeno tudi pri humanih kožnih fibroblastih (HDF) (Bain s sod., 2007). Znano je, da se geni *GADD45* inducirajo hitro po izpostavitvi širokemu spektru genotoksičnih agensov (Dickinson s sod., 2004; Ellinger-Ziegelbauer s sod., 2005; Heise s sod., 2012). Proteinski produkti teh genov igrajo osrednjo vlogo pri zaviranju celične rasti tako v odvisnosti od P53 kot neodvisno od njega (Liebermann in Hoffman, 1998; Zhan s sod., 1999) in kot odgovor na genotoksičen stres aktivirajo kontrolno točko v S fazi in na prehodu G2/M (Vairapandi s sod., 2002).

Izražanje gena *PCNA* pa se je znižalo kot odgovor na izpostavitev CYN. Protein PCNA (ang. proliferating cell nuclear antigen) reagira s številnimi proteini celičnega cikla, je glavna komponenta evkariontskega replisoma (Kelman in O'Donnell, 1995) in je vpleten v popravljanje DNK poškodb in regulacijo celičnega cikla (Maga in Hubscher, 2003). Znižalo se je tudi izražanje gena *UHRF1*, ki kodira UHRF1 (ang. ubiquitin-like with PHD and ring finger domains 1), ki reagira s proteinom PCNA (Sharif s sod., 2007) in stimulira prehod iz G1 v G2 fazo (Arita s sod., 2008).

Povišano izražanje številnih genov, kot so *CHEK1*, *CDKN1A*, *CDKN2B*, *GADD45α*, *GADD45β* ter znižano izražanje med drugimi tudi genov *PCNA* in *UHRF1* nakazuje aktivacijo kontrolnih točk celičnega cikla, njegovo ustavitev in aktivacijo popravljanja poškodb DNK.

Geni vpleteni v popravljanje DNK poškodb

Med izbranimi geni, ki so vpleteni v popravljanje DNK, smo najmočnejše povišanje izražanja (več kot 3-krat po obeh časih izpostavitve) zaznali za gen *XPC* (ang.: global genome nucleotide excision repair), ki je vpleten v popravljanje z izrezovanjem nukleotidov (NER). Povišano je bilo tudi izražanje gena *ERCC4* (več kot 2,3-krat), ki je prav tako vpleten v NER. Povišanje teh genov nakazuje na aktivacijo tega popravljalnega mehanizma, ki je odgovoren za popravljanje velikih DNK aduktov (ang.: bulky DNA adducts) in navzkrižnih povezav med verigami (Christmann s sod., 2003). XPC je transkripcijska tarča proteina P53 in se inducira kot odgovor na izpostavitev UV svetlobi,

ionizirajočemu sevanju, alkilirajočim agensom in Benzo[a]piren diol peroksidu (Amundson s sod., 2002; Wang s sod., 2003). Njegov proteinski produkt je vpleten v prepoznavanje velikih aduktov DNK (Amundson s sod., 2002). Ti rezultati podpirajo hipotezo, da CYN povzroča DNK adukte, kar narekuje že sama struktura CYN (Humpage s sod., 2000; Falconer in Humpage, 2001). CYN ima več potencialno reaktivnih skupin (sulfatna skupina, gvanidin, uracil), ki bi lahko reagirale z DNK (Falconer, 2008). Zlasti prisotnost uracilne skupine nakazuje, da bi se CYN lahko vezal z adeninom v RNK in DNK (Falconer in Humpage, 2001). Nastanek DNK aduktov zaradi vpliva CYN so pokazali *in vivo* v DNK izolirani iz jeter miši, ki so jih izpostavili CYN (Shaw s sod., 2000).

Med mehanizmom NER prihaja do prehodnih prelomov verig DNK, ki jih povzročajo strukturno specifične DNK endonukleaze, kot je XPF, ki jo kodira gen *ERCC4*. Gen *ERCC4* je bil znatno povišan tako po 12 kot po 24 h izpostavitvi CYN. Tudi ti rezultati skupaj z že znanimi podatki o CYN kažejo na to, da CYN povzroča adukte DNK, saj se ti popravljajo s pomočjo NER, pri čemer nastanejo prehodni prelomi DNK, ki jih lahko zaznamo s testom komet. S tem testom smo pokazali, da CYN povzroča poškodbe DNK pri celicah HepG2 in HPBL, test komet pa je pokazal pozitivne rezultate po izpostavitvi CYN tudi v številnih drugih študijah (Shen s sod., 2002; Humpage s sod., 2005; Bazin s sod., 2010; Bazin s sod., 2010).

Največ genov s spremenjenim izražanjem, iz skupine »popravljanje DNK poškodb«, je bilo vpletenih v popravljanje dvoverižnih prelomov DNK (DSBR), kar sovpada z rezultati testa mikrojeder in analizo kopičanja γH2AX, ki kažejo, da CYN povzroča DSB. Povišalo se je izražanje gena (*LIG4*) za ligazo IV, ki je vpletena v ponovno zlepljanje DSB med mehanizmom NHEJ (ang.: non-homologous end joining). Spremenjeno je bilo tudi izražanje genov (povišano izražanje *XRCC2*, znižano izražanje *MRE11A* in *RAD51*), ki kodirajo komponente kompleksa MRE11-Rad50-NBS1, ki stimulira uspešno ponovno zlepljanje in je vpleten v iniciacijo homologne rekombinacije (HRR) (Maser s sod., 1997).

Geni vpleteni v apoptozo in celično preživetje

Rezultati transkripcijskih sprememb pro- in antiapoptotskih genov ne omogočajo razlage ne v prid sprožitve apoptoze in ne v prid njenega zavrtja. Spremenjeno je bilo tako izražanje genov, vpletenih v intrinzično kot tudi v ekstrinzično pot apoptoze, ter genov, vpletenih v celično preživetje. Številni med njimi so transkripcijske tarče transkripcijskih faktorjev P53, s proapoptotsko funkcijo in NF-κB, s praviloma antiapoptotsko funkcijo (Pahl, 1999).

Izražanje gena *TNF*, ki kodira tumor nekrotizirajoči faktor α (TNFα), je bilo po izpostavitvi CYN od vseh izbranih genov najbolj povišano (več kot 20-krat). Vendar pa je

bilo to povečanje na račun dejstva, da se *TNF* v kontrolni populaciji ni izražal, in se je začel izražati v zmerni količini po izpostavitvi CYN, zaradi česar smo zaznali visoko relativno izražanje tega gena. TNFα je močan vnetni citokin s pomembno vlogo pri vnetnih procesih, regulaciji celične proliferacije, diferenciacije in v ekstrinzični apoptotski poti (Klefstrom s sod., 1997).

Povišano je bilo tudi izražanje drugih proapoptotskih genov iz ekstrinzične apoptotske poti, kot so *FAS* in *TNFRSF10A*, ki kodirata člana družine TNFα receptorjev (TNFR), *TRADD*, ki kodira adaptorsko molekulo, vpleteno v s FAS inducirano pot celične smrti (Van Antwerp s sod., 1998), in *CASP8*, ki kodira kaspazo 8, vpleteno v od mitohondrijske stopnje neodvisno aktivacijo kaskade efektorskih kaspaz. Vendar pa je CYN povišal tudi izražanje antiapoptotskega gena *TNFAIP3* in znižal izražanje proapototskega gena *TNFSF10*, ki sta vpletena v to pot. Slednji pozitivno stimulira sprožitev apoptoze preko aktivacije receptorjev, kot sta FAS in TRAIL, in igra tudi pomembno vlogo pri intrinzični apoptotski poti (Brunet s sod., 1999; Dijkers s sod., 2000; Modur s sod., 2002).

Intrinzična apoptotska pot je regulirana izključno s proteini iz družine BCL-2, ki lahko regulirajo tudi ekstrinzično apoptotsko pot preko proteina BID (Haupt s sod., 2003). CYN je statistično značilno spremenil izražanje 5 od 6 izbranih genov iz te družine, antiapoptotske gene *BCL2* (znižano izražanje), *BCL2L1* in *MCL1* (povišano izražanje) in proapoptotske gene *BID* (znižano izražanje) in *BAK1* (povišano izražanje). Izražanje proapoptotskega gena *BAX* je bilo rahlo povišano, vendar ni bilo statistično značilno, kar se sklada z objavljenimi rezultati, ki kažejo, da CYN statistično značilno poviša izražanje *BAX* pri celicah HepG2 in HDF šele pri višjih koncentracijah (5 µg/ml) (Bain s sod., 2007), ki so se v naših raziskavah izkazale kot citotoksične. V nasprotju smo pri HPBL opazili signifikantno povišanje izražanja tako *BAX* kot tudi *BCL2*, vendar je bilo povišanje izražanja *BCL2* močnejše od povišanja izražanja *BAX*. Razmerje med antagonisti (BCL-2, BCL-XL, MCL-1 in A-1) in agonisti apoptoze (BAX, BAK, BAD, BID in BCL-XS) naj bi odločalo o tem, ali se bo celica odzvala na signal s sprožitvijo apoptoze ali ne (Kroemer, 1997), vendar naši rezultati niso pokazali jasne prevlade ne enih ne drugih.

Med intrinzično potjo sprostitev citokroma C iz mitohondrijev aktivira apoptotski proteaze-aktivirajoči faktor-1 (APAF1), tako da nastane apoptosom, ki aktivira kaspazo 9 in posledično kaspazno kaskado (Youle in Strasser, 2008). Skupaj s citokromom C se iz mitohondrijev sprosti tudi protein SMAC (ali DIABLO), ki prav tako stimulira aktivacijo kaspaz (Du s sod., 2000; Verhagen s sod., 2000). CYN je znižal izražanje gena *APAF1* po 24 h, vendar pa je povišal izražanje gena za kaspazo 9 (*CASP9*) in *DIABLO* po obeh časih izpostavitve.

Vse apoptotske poti na koncu konvergirajo pri aktivaciji kaspaz (Klefstrom s sod., 1997; Youle in Strasser, 2008). V naši študiji je CYN poleg kaspaznih genov *CASP9* in *CASP8*

povišal tudi izražanje gena za efektorsko kaspazo *CASP3* po 24 h in znižal izražanje *CASP7* po 12 h izpostavitve .

Geni vpleteni v detoksifikacijo in metabolizem

Encimi iz družine CYP450 igrajo pomembno vlogo pri oksidativnem metabolizmu ksenobiotikov in njihovi aktivaciji. CYN je vplival na izražanje številnih genov CYP450, kar podpira domnevo, da so vpleteni v njegovo aktivacijo. S klasičnim QPCR smo pokazali, da CYN poviša izražanje genov *CYP1A1* in *CYP1A2* pri celicah HepG2 kot tudi pri HPBL. V nasprotju s temi rezultati je transkriptomska analiza s PCR čipi pokazala, da se izražanje *CYP1A1* močneje poviša po 12 h izpostavitvi, izražanje *CYP1A2* pa se ni statistično značilno spremenilo. Možna razlaga za dobljene razlike je uporaba različnih sond in PCR kemije (TaqMan in Sybr Green) in pa dejstva, da je izražanje *CYP1A2* v celicah HepG2 zelo šibko, tako da že majhne variacije lahko pomenijo veliko spremembo. Opazili smo tudi povišano izražanje *CYP1B1* po 12 h izpostavitve, kar kaže na njegovo vpletenost v aktivacijo CYN. Zanimivo je, da je bilo med geni CYP450 najbolj povišano izražanje prav genov *CYP1A1* in *CYP1B1* pri celicah HepG2 tudi po izpostavitvi znanemu prokarcinogenu benzo[a]pirenu (BaP) (Song s sod., 2012).

Številnim CYP450 genom se je po izpostavitvi CYN izražanje znižalo. Statistično značilno znižanje smo opazili za gene *CYP2A6* (12 h), *CYP2F1* (12 in 24 h), *CYP2A13*, *CYP3A43* in *CYP3A7* (24 h). Mehanizmi vpleteni v zaviranje izražanja CYP450 niso poznani. Njihovo izražanje se lahko zniža po izpostavitvi številnim kemikalijam (Riddick s sod., 2003), kot patofiziološki odziv na stresne signale, ali odziv prilagajanja oksidativnemu stresu (Morgan, 2001) ter med akutnimi fazami infekcij in vnetnimi procesi, pri katerih so vpleteni NF-κB, TNF-α in drugi vnetni citokini (Ke s sod., 2001; Morgan, 2001; Riddick s sod., 2004). Tako bi opaženo povišanje genov *NFKB1* in *TNF* lahko bilo povezano z znižanim izražanjem CYP450 genov.

CYN je spremenil tudi izražanje genov, ki kodirajo metabolične encime II. faze. Izpostavitev CYN je povzročila povišanje izražanja genov *UGT1A6* in *UGT1A1*, ki kodirata encima UDP-glukuronozil trasferazi (12 h) in *GSTM3*, ki kodira glutation S-transferazo mu 3 (12 in 24 h). Po 24 h izpostavitvi smo opazili tudi povišano izražanje *NAT1*, ki kodira N-acetil transferazo, in znižano izražanje *GSTM2*, *GSTA2*, *GNMT* (glicin N-metiltransferaza) in *SULT1A1* (sulfotransferaza). Konjugacija z metaboličnimi encimi II. faze velja za detoksifikacijsko stopnjo metabolizma ksenobiotikov, vendar lahko v določenih situacijah privede do aktivacije in povišane toksičnosti (Schilter s sod., 1993; Hinson in Forkert, 1995; Rushmore in Tony Kong, 2002). Do sedaj ni podatkov o tem, da bi v aktivacijo ali detoksifikacijo CYN bili vpleteni encimi II. faze. Rezultati transkriptomske analize nakazujejo, da so v metabolizem oz. detoksifikacijo CYN poleg CYP450 encimov vpleteni tako metabolični encimi II. faze, kot tudi drugi encimi I. faze,

saj je CYN povišal tudi izražanje gena za aldehid dehidrogenazo 1A2 (*ALDH1A2*) in karboksilesterazo 2 (*CES2*) ter znižal izražanje *CES1*.

Razen že omenjenih genov se ni spremenilo izražanje drugih izbranih genov glutation S-transferaz (GST). GST sodelujejo v procesu detoksifikacije, ki deluje preko konjugacije snovi z glutationom. Če CYN, kot je bilo predpostavljeno, zavira sintezo glutationa (Runnegar s sod., 1995; Humpage s sod., 2005), to lahko vodi v oksidativni stres, čemur v prid kaže povišano izražanje gena *GCLC*, ki kodira poglavitni encim vpleten v *de novo* sintezo glutationa, γ-glutamilcistein sintetazo (Krzywanski s sod., 2004), in gena za katalazo, *CAT*, ki kodira encim, ki katalizira pretvorbo H_2O_2 v vodo. CYN je povišal tudi izražanje tioredoksin reduktaze (*TXNRD1*), ki je potrebna za efektivno vezavo transkripcijskih faktojev občutljivih na redoks stanje, na DNK, vključno z P53 in NF-κB (Hayashi s sod., 1993; Ueno s sod., 1999; Arner in Holmgren, 2006). Vendar pa nespremenjeno izražanje številnih drugih izbranih genov, ki se odzovejo na oksidativni stres (*NOS2* in *SOD1*), in drugih genov, vpletenih v kroženje glutationa (*GSTs*, *GSS*, *GSR*, *GPXs* in *GGT1*), skupaj z negativnimi rezultati modificiranega testa komet kaže, da CYN ne povzroča obsežnejšega oksidativnega stresa.

3.1.3 Metabolična aktivacija CYN

Raziskave genotoksičnosti kažejo, da je CYN pro-genotoksin, kar pomeni, da so za njegove genotoksične lastnosti najverjetneje odgovorni njegovi metabolični produkti. Genotoksični učinki CYN v *in vitro* raziskavah so omejeni na testne sisteme, ki so metabolično kompetentni. Tako genotoksičnih učinkov niso opazili pri celični liniji CHO-K1 (Fessard and Bernard 2003; Lankoff, Wojcik et al. 2007) in drugih sistemih, ki imajo nizko metabolično aktivnost (Fessard in Bernard, 2003; Lankoff s sod., 2007; Bazin s sod., 2010). Domnevno so v aktivacijo CYN vpleteni metabolični encimi iz družine CYP450, vendar pa ni znano kateri. Vpletenost CYP450 encimov so pokazali posredno, z uporabo široko spektralnih zaviralcev teh encimov, ki znižajo toksične (Runnegar s sod., 1995; Norris s sod., 2002; Froscio s sod., 2003) in genotoksične učinke CYN (Humpage s sod., 2005; Bazin s sod., 2010). Kot smo že omenili v prejšnjem poglavju, ni podatkov o tem, da bi v njegovo presnavljanje bili vpleteni tudi drugi encimi I. in II. faze. Rezultati transkriptomske analize pa nakazujejo, da so vpleteni številni metabolični geni.

V doktorski disertaciji smo napravili dodatne teste, za potrditev domneve, da se CYN presnavlja. Rezultati standardne analize stabilnosti CYN v prisotnosti humanih hepatocitov so pokazali, da se CYN ne presnavlja pod testnimi pogoji. Rezultati niso presenetljivi, saj so bili hepatociti izpostavljeni CYN za največ 90 min, kar pa je prekratek čas, da bi CYN lahko prešel v celico. S testom mikrosomalne stabilnosti pa smo potrdili, da se CYN

srednje intenzivno presnavlja z encimi I. faze in ima intrinzični očistek (16,9 µl/min/mg) in razpolovni čas pri 82 min (Tabela 2).

3.1.4 Vpliv CYN na apoptozo

V literaturi ne najdemo veliko podatkov o vplivu CYN na sprožitev apoptoze. Tri študije poročajo o sprožitvi apoptoze pri CHO-K1 celicah, izpostavljenih CYN (Fessard in Bernard, 2003; Lankoff s sod., 2007; Gácsi s sod., 2009). V vseh treh publikacijah poročajo o rahlih spremembah, povezanih z apoptozo, vendar šele pri višjih koncentracijah, kot smo jih uporabili v doktorski disertaciji. Prav tako celice CHO-K1, kot smo že omenili, niso primerljive s HepG2, saj imajo nizko metabolično aktivnost. Tako je uporaba CHO-K1 celic za študijo mehanizmov CYN vprašljiva.

Kot smo opisali v poglavju 3.1.2, rezultati transkriptomske študije kažejo na aktivacijo ekstrinzične kot tudi intrinzične apoptotske poti, vendar ni jasno, ali prevladujejo pro- ali antiapoptotski signali.

Rezultati analize vpliva CYN na mitohondrijski membranski potencial (MMP) so pokazali, da CYN povzroča mitohondrijsko hiperpolarizacijo (MHP). MHP je najzgodnejša sprememba, povezana s številnimi apoptotskimi potmi, vendar je reverzibilen pojav in ni povezana izključno z apoptozo (Perl s sod., 2004). Po eni strani poročajo, da predstavlja aktivno stopnjo v procesu apoptoze (Poppe s sod., 2001), po drugi pa so predpostavili, da apoptozo zavira (Liang s sod., 1999; Iijima, 2006). Vloga MHP še ni poznana. Sprememba MMP, ki smo jo opazili, potrjuje rezultate transkriptomske analize, kjer je bilo spremenjeno izražanje številnih genov iz družine *BCL-2*, katerih proteinski produkti vplivajo na MMP (Adams in Cory, 2007; Chipuk in Green, 2008).

CYN je povzročil tudi spremenjeno izražanje genov za kaspaze 3 in 7, vendar teh sprememb nismo potrdili na proteinskem nivoju, saj CYN ni povzročil statistično značilne spremembe aktivnosti teh dveh kaspaz. Poleg tega smo z barvanjem z Aneksinom V in propidijevim iodidom (PI) pokazali, da CYN ne vpliva statistično značilno na odstotek zgodnje apoptotskih, pozno apoptotskih ali nekrotičnih celic v populaciji. Prav tako pri testiranih pogojih ne povzroča nekroze, saj ni povzročil povečanega puščanja laktat dehidrogenaze (LDH).

Vsi rezultati skupaj nakazujejo, da CYN pri celicah HepG2 povzroča stres, ki sproži začetne apoptotske signale, vendar apoptoze v končni fazi pri testiranih pogojih ne povzroča.

3.1.5 Vpliv CYN na celični cikel in celično proliferacijo

Predpostavili smo, da CYN zaradi svoje genotoksične aktivnosti in sposobnosti ireverzibilnega zaviranja sinteze proteinov (Froscio et al. 2003; Humpage et al. 2005; Norris et al. 2002; Runnegar et al. 1995) vpliva na celični cikel.

Rezultati testa MTT s podaljšano izpostavitvijo so pokazali, da CYN statistično značilno od koncentracije in časa odvisno zniža živost celic HepG2. Znižana živost celic, ki jo izmerimo s testom MTT, je lahko posledica celične smrti, zmanjšane aktivnosti celic (zmanjšana aktivnost mitohondrijskih dehidrogenaz) ali zmanjšane celične proliferacije.

Opisane spremembe smo opazili tudi pod mikroskopom, kjer je pri celicah izpostavljenim CYN (0,5 µg/ml) prišlo do zmanjšane gostote celic v primerjavi s kontrolo in morfoloških sprememb. Po daljšem času izpostavitve (72 in 96 h) smo pri nekaterih celicah opazili spremembe podobne znakom apoptoze, kot so brstenje celične površine, zaokrožanje celic in rahlo povišanje števila plavajočih celic.

S testom puščanja LDH smo izključili, da je bila zmanjšana živost celic na račun nekroze. Opazili smo celo zmanjšanje puščanja LDH, vendar pa se je znižala tudi količina celokupne LDH, tako da se razmerje ni značilno spremenilo.

Rezultati kažejo, da CYN pri testiranih pogojih ni citotoksičen za celice in da je zmanjšana živost celic, ki smo jo izmerili s testom MTT, posledica zmanjšane celične proliferacije in ne celične smrti. To smo potrdili z imunocitokemijsko analizo kazalca celične proliferacije, proteina Ki67. Protein Ki67 je strogo povezan izključno s celično proliferacijo, saj je prisoten le med aktivnimi fazami celičnega cikla (G1, S, G2 in M) in ga ne najdemo v mirujočih celicah (G0) (Michael in Oren, 2002).

Statistično značilno znižano število celic pozitivnih na Ki67 smo opazili že po 24 h izpostavitvi CYN pri koncentraciji 0,5 µg/ml, po daljši izpostavitvi (72 in 96 h) pa tudi pri nižjih koncentracijah. Glede na to, da je CYN zaviralec sinteze proteinov, bi lahko bilo zmanjšanje Ki67 proteina direktna posledica tega mehanizma (Bruno and Darzynkiewicz 1992). Vendar ker je znižanje števila na Ki67 pozitivnih celic sovpadalo z znižanjem živosti celic in zmanjšano gostoto celic, ki smo jo opazili pod mikroskopom, najverjetneje odraža znižano celično proliferacijo. Prav tako ti rezultati sovpadajo z rezultati testa mikrojeder, kjer smo pokazali znižan jedrni delitveni indeks tako pri celicah HepG2 kot pri HPBL. Seveda pa doprinosa zaviranja sinteze proteinov k zmanjšanemu izražanju proteina Ki67 ne moremo popolnoma izključiti.

Rezultati naše študije o vplivu CYN na celično proliferacijo se skladajo z rezultati drugih objav. Fesard in Bernard (2003) (Fessard in Bernard, 2003) in Lankoff s sod. (2007)

(Lankoff s sod., 2007) poročajo, da CYN povzroča zmanjšano število mitotičnih celic in znižan mitotični indeks ter zmanjšano proliferacijo pri celicah CHO-K1. Humpage s sod. (2000) (Humpage s sod., 2000) pa je opazil od koncentracije odvisno zaviranje celične delitve pri limfoblastoidni celični liniji WIL2-NS po izpostavitvi CYN.

Analiza celičnega cikla s pretočno citometrijo je pokazala, da CYN vpliva na celični cikel že po 24 h izpostavitve. Opazili smo statistično značilno povišanje deleža celic v G0/G1 fazah in znižanje deleža celic v G2/M fazah, kar bi lahko pomenilo, da CYN preprečuje mirujočim celicam vstop v nov cikel ali preprečuje prehod celic v S fazo. Rezultati se skladajo tudi s transkriptomsko analizo s QPCR čipi, kjer smo zaznali spremenjeno izražanje številnih genov, ki sprožijo aktivacijo kontrolnih točk in ustavitev celičnega cikla v G fazi (glej poglavje: 3.1.2). Že več kot desetletje je znano, da poškodbe DNK povzročajo ustavljanje cikla v G1 fazi preko P53 in $P21^{WAF1/CIP1}$ (za pregled glej (Ko in Prives, 1996; Levine, 1997)), kar se prav tako sklada z rezultati transkriptomske analize.

Po daljši izpostavitvi (72 in 96 h) je prišlo do statistično značilnega povišanja odstotka celic v S fazi, hkrati pa se je znižal odstotek v G0/G1, kar nakazuje na ustavitev celičnega cikla v S fazi po daljši izpostavitvi CYN. V S fazi so 3 kontrolne točke: kontrolna točka podvajanja in S/M kontrolna točka, ki se odzivata na napake pri podvajanju DNK, in kontrolna točka znotraj S faze, ki je neodvisna od podvajanja DNK in se inducira kot odgovor na nastanek DSB(Bartek s sod., 2004). Rezultati se skladajo z analizo kopičenja γH2A.X, kjer smo opazili nastajanje DSB po podaljšanem času izpostavitve (72 h). Da CYN povzroča DSB nakazujejo tudi rezultati transkriptomske analize in testa mikrojeder. Slednji je pokazal tudi povišano število nastajanja NBUD in NPB pri celicah HepG2 in HPBL po izpostavitvi CYN. Nastanek teh celičnih struktur je posledica amplificirane DNK (Shimizu s sod., 1998) ali kromatira, katerega podvajanje v S fazi ni bilo uspešno (Yankiwski s sod., 2000) in ga celica odstrani iz jedra, ali pa kromosomskih premestitev, napak pri popravljanju ali fuzij telomer (Fenech, 2000). Humpage s sod. (2000) (Humpage s sod., 2000Humpage s sod., 2000) je pokazal, da CYN lahko deluje tako klastogeno kot aneugeno, domneva, da lahko CYN med delitvijo celic deluje na delitveno vreteno ali centromere in tako lahko povzroči izgubo celega kromosoma. Naši rezultati in možno aneugeno delovanje CYN (Humpage s sod., 2000) kažejo, da lahko CYN povzroča tudi napake pri podvajanju DNK, ki bi prav tako lahko sprožile aktivacijo kontrolnih točk v S fazi.

3.2 SKLEPI

V sklopu doktorske disertacije smo prišli do naslednjih ugotovitev:

- Potrdili smo hipotezo, da vsi trije izbrani cianotoksini povzročajo poškodbe DNK pri necitotoksičnih koncentracijah in delujejo genotoksično.
    - CYN pri koncentracijah do vključno 0,5 µg/ml ni toksičen za celice HepG2 in HPBL, saj ne vpliva na živost celic po 24 h izpostavitvi, kar določamo s testom MTT.
    - CYN povzroča poškodbe DNK, ki jih zaznamo s testom komet pri celicah HepG2 in HPBL.
    - CYN poviša nastajanje MNi, NBUD in rahlo poviša nastajanje NPB pri celicah HepG2 in HPBL ter zniža jedrni delitveni indeks.
    - CYN po podaljšanem času izpostavitve (72 h) povzroča kopičenje γH2A.X, kar kaže na nastanek DSB.
    - CYN (0,5 µg/ml) po 12 in 24 h izpostavitvi celic HepG2 povzroča spremembo v izražanju številnih izbranih genov, ki se odzivajo na genotoksični stres. Spremembe v izražanju nakazujejo na to, da CYN:
        - sproži takojšnji-zgodnji odziv, saj močno poviša izražanje genov iz družin *FOS* in *JUN*;
        - sproži aktivacijo signalnih poti P53 in NFκβ, saj vpliva na izražanje številnih genov, ki so tarče teh dveh transkripcijskih faktorjev;
        - sproži aktivacijo kontrolnih točk in ustavitev celičnega cikla, saj močno poviša izražanje genov *GADD45A*, *GADD45B*, *CDKN1A*, *CDKN2B* in *CHEK1*;
        - sproži DNK popravljalne mehanizme DSBR in NER.
    - CYN (0,5 µg/ml) po 24 h izpostavitvi pri HPBL povzroča spremembo v izražanju genov vpletenih v odgovor na poškodbe DNK (*P53*, *MDM2*, *GADD45α*), v regulacijo apoptoze (*BAX* in *BCL2*) in v odziv na oksidativni stres (*GPX1*, *GSR*, *GCLC* in *SOD1*).

- MCLR ni toksičen za HPBL pri nobeni od testiranih koncentracij po 24 h izpostavitvi do vključno 10 μg/ml.

- MCLR pri HPBL povzroča od koncentracije in časa odvisno nastajanje DNK poškodb, ki jih zaznamo s testom komet.

- MCLR ne vpliva na nastajanje MNi, NBUD in NPB pri HPBL. Prav tako ne zniža jedrnega delitvenega indeksa.

- MCLR (1 μg/ml) povzroča povišano izražanje genov vpletenih v odgovor na poškodbe DNK (*P53*, *MDM2*, *GADD45α*, *CDKN1A*) pri HPBL po 24 h izpostavitve.

- NOD ni toksičen za celice HepG2 pri nobeni od testiranih koncentracij po 24 h izpostavitvi do vključno 10 μg/ml.

- NOD povzroča poškodbe DNK, ki jih zaznamo s testom komet pri celicah HepG2 le pri višjih koncentracijah (1 μg/ml).

- NOD povzroča le manjše spremembe izražanja genov, vpletenih v odgovor na DNK poškodbe (*P53*, *CDKN1A*, *GADD45α* in *MDM2*).

- Potrdili smo hipotezo, da oksidativni stres ni vpleten v genotoksično delovanje CYN, vendar igra pomembno vlogo pri MCLR in NOD.

  - Kljub temu, da CYN pri celicah HepG2 povzroča rahlo povišanje nastajanja ROS, ne povzroča obsežnejših oksidativnih poškodb DNK, ki jih zaznamo z modificiranim testom komet z encimom Fpg in ne povzroča poglavitnih sprememb v izražanju genov, vpletenih v odgovor na oksidativni stres.

  - MCLR povzroča oksidativne poškodbe DNK pri HPBL že pri nizkih koncentracijah (0,1–1 μg/ml), vendar daljših časih izpostavitve (24 h).

  - NOD povzroča povišano nastajanje ROS in oksidativne poškodbe DNK že pri nizkih koncentracijah (0,01–1 μg/ml) in kratkih časih izpostavitve (4 h), s podaljšanim časom izpostavitve pa količina poškodb počasi upada.

- Potrdili smo hipotezo, da se CYN presnavlja z encimi iz družine CYP450.

- CYN se v prisotnosti humanih hepatičnih mikrosomov presnavlja s srednjo vrednostjo intrinzičnega očistka in razpolovnim časom 82 min.

- CYN povzroča povišano izražanje genov za metabolične encime *CYP1A1* in *CYP1A2* pri celicah HepG2 in HPBL, kar kaže na njihovo vključenost v metabolično pretvarjanje CYN.

- Rezultati transkriptomske analize na celicah HepG2 izpostavljenih CYN za 12 in 24 h nakazujejo vpletenost še drugih encimov [metaboličnih encimov I. (*CYP1A1*, *CYP1B*, *ALDH1A2* in *CES2*) in II. faze (*UGT1A6*, *UGT1A1*, *NAT1* in *GSTM3*)].

- Ovrgli smo hipotezo, da CYN povzroča apoptozo, saj apoptoze nismo zasledili pri genotoksičnih koncentracijah pri celicah HepG2.

- CYN pri testiranih pogojih tudi ne povzroča nekroze, saj nismo zaznali povišanega puščanja LDH.

- Potrdili smo hipotezo, da CYN vpliva na celični cikel, saj ustavlja celični cikel pri celicah HepG2 v G0/G1 fazi po krajšem času izpostavljenosti (24 h) in v S fazi po daljšem času (72 in 96 h).

- CYN povzroča zmanjšano celično proliferacijo pri celicah HepG2, ki jo zaznamo posredno preko kazalca celične proliferacije, Ki67.

- Noben od toksinov (CYN, NOD in MCLR) ni bakterijski mutagen, saj pri testiranih pogojih ni izpolnil kriterijev mutagenosti, kot jih določamo s testom Ames.

4 POVZETEK (SUMMARY)

4.1 POVZETEK

Cianotoksini, ki jih proizvajajo cianobakterije v površinskih vodnih zajetjih, predstavljajo vse bolj pereč problem po vsem svetu zaradi vse pogostejšega pojavljanja cianobakterijskih cvetenj kot posledice klimatskih sprememb in eutrofikacije voda. Preučevali smo genotoksično delovanje predstavnikov najpogostejših cianotoksinov v sladkih in brakičnih vodah: MCLR, NOD in CYN; s posebnim poudarkom na najslabše preučenemu cianotoksinu CYN. V raziskavi smo uporabili nizke, za okolje relevantne koncentracije, ki niso citotoksične za celice.

Čeprav nobeden od preučevanih cianotoksinov ni bakterijski mutagen, so vsi trije toksini povzročali genotoksične učinke v izbranih humanih testnih sistemih.

Genotoksično delovanje CYN in celični odziv na izpostavitev temu toksinu smo testirali na različnih celičnih nivojih. Pri celicah HepG2 smo nastanek poškodb DNK po izpostavitvi CYN analizirali s testom komet, s testom mikrojeder in detekcijo γH2A.X s pretočno citometrijo. Poleg tega smo s kvantitativnim PCR v realnem času in QPCR čipi analizirali spremembe v izražanju izbranih genov, ki so vključeni v odziv na poškodbe DNK in odziv na genotoksičen stres. Ugotovili smo, da CYN pri necitotoksičnih koncentracijah povzroča poškodbe DNK po 12 in 24 h izpostavljenosti, poviša pogostost MNi, NBUD in NPB po 24 h izpostavljenosti in povzroča DSB po podaljšanem času izpostavitve (72 h). CYN je povišal izražanje s P53 reguliranih genov *CDKN1A*, *GADD45α* in *MDM2*, kar nakazuje na sprožitev signalizacijske poti P53. To je potrdila tudi toksikogenomska analiza izražanja genov s QPCR čipi. Dodatno smo ugotovili, da CYN poviša izražanje genov takojšnjega-zgodnjega odziva iz genske družine *FOS* in *JUN*, rezultati alternacij v izražanju tarčnih genov pa kažejo tudi na sprožitev NF-κB signalizacije.

Pri HPBL smo pokazali, da CYN povzroča nastanek DNK prelomov ter od koncentracije in časa povišan nastanek MNi in NBUD, medtem ko se je število NPB le rahlo povišalo. CYN je pri HPBL povzročil tudi spremembe v izražanju genov vpletenih v odziv na poškodbe DNK (*P53*, *MDM2*, *GADD45a*), gene vpletene v regulacijo apoptoze (*BCL-2* in *BAX*) kot tudi v odziv na oksidativni stres (*GPX1*, *SOD1*, *GSR*, *GCLC*).

Prav tako je tudi MCLR pri HPBL povzročil od koncentracije in časa odvisno povišanje nastanka poškodb DNK, ki smo jih merili s testom komet. V nasprotju s testom mikrojeder nismo zaznali sprememb v pojavljanju MNi, NBUD ali NPB po 24 h izpostavitve. Pri izoliranih HPBL, ki so bili izpostavljeni MCLR, smo po razgradnji DNK z encimom Fpg zaznali večje število prelomov DNK verig kot pri neencimsko razgrajeni DNK. Ti rezultati potrjujejo, da MCLR povzroča oksidativne poškodbe DNK. Po 24 h izpostavitve MCLR je

prišlo do povišanega izražanja genov vpletenih v odziv na DNK poškodbe (*P53*, *MDM2*, *GADD45α*, *CDKN1A*), oksidativni stres (*CAT*, *GPX1*, *SOD1*, *GSR*, *GCLC*) in gena, vključenega v apoptozo (*BAX*). Ti rezultati podajajo dokaze, da je MCLR posredno genotoksičen agens, ki deluje preko sprožitve oksidativnega stresa, in da so tudi limfociti tarča toksičnih učinkov MCLR.

Rezultati kažejo, da je tudi pri NOD glavni mehanizem genotoksičnosti nastanek oksidativnega stresa. Pri celicah HepG2 je NOD povzročal od koncentracije in časa odvisno povišanje ROS, ki smo jih merili s sondo DCFH-DA. Modificiran test komet je pokazal, da se je število prelomov znatno povišalo po encimski razgradnji s Fpg. Najvišji nivo oksidativnih DNK poškodb smo opazili po 4 h izpostavitve, vendar je ostal statistično značilno povišan tudi po 12 in 24 h izpostavitve koncentracijama 1 in 0,1 µg/ml. V nasprotju smo brez razgradnje s Fpg opazili le manj obsežne poškodbe DNK po izpostavitvi NOD. Prav tako pa je toksin vplival na izražanje genov vpletenih v odziv na poškodbe DNK le v manjši meri.

Da bi preverili, ali tudi CYN vsaj delno povzroča poškodbe DNK preko sprožitve oksidativnega stresa, smo merili nastajanje ROS in nastajanje oksidativnih DNK poškodb pri celicah HepG2. CYN je statistično značilno povišal nastajanje ROS. Vendar pa kljub nastanku ROS, po kratkem času izpostavitve (4 h), nismo zaznali povišane ravni oksidiranih purinov. Po 12 in 24 h izpostavitve je CYN povzročil tako povišano raven poškodb DNK po encimski razgradnji kot brez nje, kar kaže na to, da oksidativni stres nima znatne vloge pri nastanku poškodb DNK. Na to so kazali tudi toksikogenomski rezultati, kjer je CYN izmed številnih izbranih genov, vpletenih v odziv na oksidativni stres, povišal izražanje le pri genih *CAT* in *TXNRD1*.

Poleg tega smo analizirali sprožitev apoptoze po izpostavitvi CYN. Vzorci deregulacije pro- in antiapoptotskih genov toksikogenomske študije niso podali jasnega odgovora, ali CYN povzroča apoptozo. Rezultati kažejo, da se sprožita intrinzična kot tudi ekstrinzična apoptotska pot, vendar pa je bilo spremenjeno izražanje tudi pri številnih antiapoptotskih genih. Toksin je pri celicah HepG2 statistično značilno povišal MMP po 12 in 24 h izpostavitve, kar so nakazovali tudi transkriptomski podatki. V nasprotju do spremembe v aktivnosti kaspaz 3 in 7 ni prišlo. Prav tako nismo opazili sprememb v številu apoptotskih celic, kar smo določali z barvanjem z Annexin V in PI. Zaključimo lahko, da CYN ne povzroča apoptoze pri genotoksičnih koncentracijah, kar poveča tveganje za ljudi in živali posebej pri dolgodobni izpostavitvi nizkim koncentracijam, saj poškodovane, potencialno škodljive celice, niso odstranjene.

Po dolgotrajnejši izpostavitvi CYN smo opazili morfološke spremembe celic HepG2 in znižanje njihove živosti v odvisnosti od koncentracije in časa. Le rahlo in statistično neznačilno puščanje LDH smo opazili po podaljšanem času izpostavitve (72 in 96 h), kar

kaže, da je znižana živost celic po izpostavitvi CYN na račun znižane celične proliferacije in ne smrti. To smo potrdili z imunocitokemijsko analizo proliferacijskega kazalca Ki67. Opazili smo, od koncentracije in časa odvisno, znižanje celic pozitivnih na ta kazalec. Analiza celičnega cikla s pretočno citometrijo je pokazala, da CYN povzroča ustavitev celičnega cikla v G0/G1 fazi po 24 h izpostavitve in v S fazi po podaljšanem času (72 in 96 h). S tem se skladajo tudi rezultati transkriptomske analize, kjer smo opazili močno povišanje izražanja genov, ki so inducibilni z DNK poškodbami (*GADD45A* in *GADD45B*), inhibitorjev od ciklinov odvisnih kinaz (*CDKN1A* in *CDKN2B*), kinaze kontrolne točke 1 (*CHEK1*) in genov, ki sodelujejo v popravljalnih mehanizmih DNK poškodb (*XPC*, *ERCC4* in drugi), ki nakazuje na ustavitev celičnega cikla in sprožitev popravljalnih mehanizmov DNK (DSBR in NER).

Da bi potrdili domnevo, da je CYN pro-genotoksin, smo želeli dokazati, da se presnavlja z metaboličnimi encimi. S testom mikrosomalne stabilnosti smo potrdili, da se CYN srednje intenzivno presnavlja z encimi I. faze, ima intrinzični očistek (16,9 µl/min/mg) in razpolovni čas pri 82 min. Rezultati standardnega QPCR so pokazali, da sta v metabolično aktivacijo CYN pri celicah HepG2 in HPBL vpletena encima CYP1A1 in CYP1A2, rezultati transkriptomske študije pa so dodatno nakazali vpletenost drugih metaboličnih genov I. faze (CYP1A1, CYP1B, ALDH1A2 in CES2) in II. faze (UGT1A6, UGT1A1, NAT1 in GSTM3).

4.2 SUMMARY

Cyanobacterial toxins produced by freshwater cyanobacteria are increasingly recognized as a threat to human health due to the increasing occurrence of cyanobacterial blooms as a result of climate change and eutrophication of water bodies worldwide. We studied the genotoxic effects of representative congeners of cyantoxins most commonly found in fresh and brackish waters, MCLR, NOD and CYN, with special emphasis on the least studied cyanotoxin CYN. In the experiments low environmentally relevant concentrations that are not cytotoxic to cells were used.

Although none of the studied cyanotoxins is a bacterial mutagen, all three toxins caused genotoxic effects in selected human cell test systems.

Genotoxic effects of CYN and the cellular response to the exposure to this toxin was examined at different levels. In HepG2 cells, the formation of DNA damage after CYN exposure was assessed by the comet assay, the micronucleus assay and flow-cytometric detection of γH2A.X. In addition, we analyzed changes in the expression of selected genes involved in the response to DNA damage and response to genotoxic stress, using quantitative real-time PCR and QPCR arrays. We found that non-cytotoxic CYN concentrations caused DNA damage after 12 and 24 hours of exposure, increased incidence of MNi, NBUDs and NPBs after 24 hours of exposure and caused DSBs after ptolonged exposure (72 h). CYN increased expression of P53 downstream-regulated genes *CDKN1A*, *GADD45α* and *MDM2*, which indicates induction of P53 signalling. This was also confirmed by the toxicogenomic analysis of gene expression by QPCR arrays. In addition, we found that CYN induced the immediate-early response genes from the FOS and JUN gene families, and induction of NF-κB signalling was indicated.

In HPBLs CYN induced DNA breaks in a dose and time-dependent manner and increased the frequency of MNi and NBUD, while the number of NPB was only slightly increased. It caused changes in the expression of genes involved in the response to DNA damage (*P53*, *MDM2*, *GADD45*), genes involved in the regulation of apoptosis (*BCL-2* and *BAX*), as well as in response to oxidative stress (*GPX1*, *SOD1*, *GSR*, *GCLC*).

Also MCLR caused dose and time-dependent increase in DNA damage in HPBLs. While no changes in the occurrence of MNi, NBUDs or NPB were detected after 24-h exposure. After Fpg digestion, increased number of DNA breaks was observed, which confirms that MCLR caused oxidative DNA damage. After 24 hours of exposure to MCLR increased expression of genes involved in the response to DNA damage (*P53*, *MDM2*, *GADD45α* and *CDKN1A*), oxidative stress (*CAT*, *GPX1*, *SOD1*, *GSR*, *GCLC*) and apoptosis (*BAX*) was observed. These results provide evidence for indirect genotoxicity of MCLR, acting

through induction of oxidative stress, and that lymphocytes can be a target of MCLR genotoxic activity.

Our results show that also the main mechanism of NOD genotoxicity is the induction of oxidative stress. In HepG2 cells, NOD caused dose and time-dependent increase in ROS production, which was measured using the DCFH-DA probe. The modified comet assay showed that the number of DNA breaks increased significantly after Fpg digestion. The highest level of oxidative DNA damage was observed after 4 h of exposure, but remained significantly elevated even after 12 and 24 h of exposure to concentrations 1 and 0.1 µg/ml. In contrast, without Fpg digestion only slight DNA damage after exposure to NOD was detected, and the toxin had only slight effect on the expression of genes involved in the response to DNA damage.

To determine whether also CYN causes DNA damage at least partly through the induction of oxidative stress, we measured the formation of ROS and the formation of oxidative DNA damage in HepG2 cells. CYN significantly increased the formation of ROS. However, after a short exposure time (4 h) no induction of oxidized purines was detected. After 12 and 24 h of exposure CYN caused elevated levels of DNA strand breaks without enzymatic digestion. Folowing Fpg digestion the level of DNA damage did not increase, showing that oxidative stress does not play a significant role the genotoxic activity of CYN in HepG2 cells. This correlates with the results from the toxicogenomic analysis, where CYN, from a number of selected genes involved in the response to oxidative stress, increased only the expression of *CAT* and *TXNRD1*.

In addition, we analyzed the induction of apoptosis after exposure to CYN. The deregulation patterns of pro-and anti-apoptotic genes in the toxicogenomic study did not provide a clear answer, whether CYN caused apoptosis. While induction of the intrinsic and extrinsic apoptotic pathway was indicated, also several anti-apoptotic genes were differentially expressed. CYN significantly increased MMP after 12 and 24 h of exposure in HepG2 cells, which was also indicated by the toxicogenomic data. No changes in the activity of caspases 3 and 7 were detected and there were also no changes in the number of apoptotic cells, determined by Annexin V and PI staining. The results showed that CYN did not cause apoptosis at genotoxic concentrations, which intensifies the hazard of CYN especially at long-term exposure to low levels, as damaged, potentially harmful cells, are not removed.

After prolonged exposure to CYN morphological changes of HepG2 cells and dose and time-dependant reduction in viability were observed. Only a slight and not significant LDH leakage was observed after prolonged exposure (72 and 96 h), suggesting that the reduced cell viability after CYN exposure was the consequence of reduced cell growth and not death. This was confirmed by immunocytochemical analysis of the proliferation marker

Ki67, as we observed time and dose-dependent decrease of Ki67 positive cells. Analysis of the cell cycle by flow cytometry showed that CYN caused cell cycle arrest in G0/G1 phase after 24 h of exposure and in the S phase after a prolonged exposure (72 and 96 h). The results are also consistent with the toxicogenomic analysis. We observed a strong up-regulation of DNA damage inducible genes (*GADD45α* and *GADD45β*), cyclin dependent kinases inhibitors (*CDKN1A* and *CDKN2B*), checkpoint kinase 1 (*CHEK1*) and genes involved in DNA damage repair mechanisms (*XPC*, *ERCC4* and others), which indicated induction of cell cycle arrest and DNA damage repair mechanisms (DSBR and NER).

To confirm the assumption that CYN is a pro-genotoxin, we wanted to prove that CYN was metabolized by metabolic enzymes. The microsomal stability assay confirmed that CYN is moderately metabolised by phase I enzymes, with an intrinsic clearance of 16.9 ml/min/mg and a half-life of 82 min. The QPCR results showed that CYP1A1 and CYP1A2 are involved in the metabolic activation of CYN in HepG2 cells and HPBL, while the transcriptomic analyses have further indicated involvement of other phase I (CYP1A1, CYP1B, ALDH1A2 and CES2) and phase II (UGT1A6, UGT1A1, GSTM3 and NAT1) metabolic enzymes.

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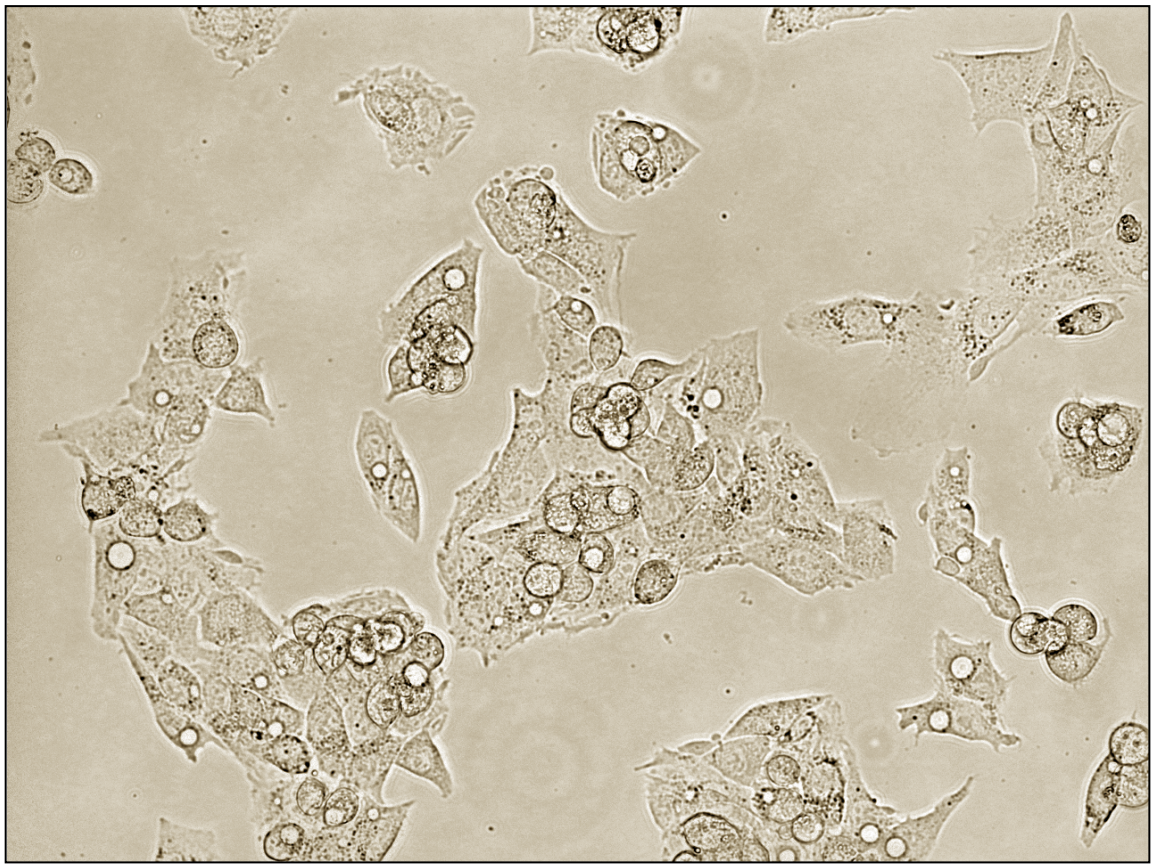
PRILOGA A

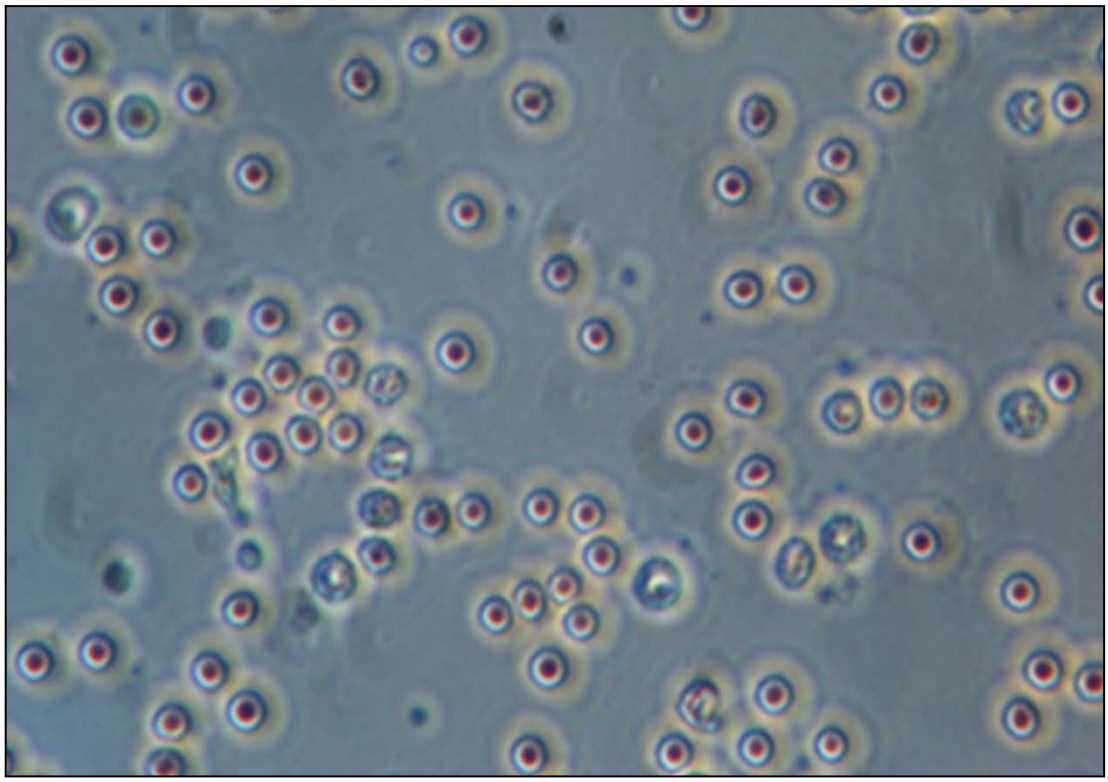
Celice HepG2 pod faznim kontrastom.



Povečava 460 x.

PRILOGA B

Primarni humani limfociti iz periferne krvi (HPBL) pod svetlobnim mikroskopom.



Povečava 400 x. (Slika: Goran Gajski)

PRILOGA C

Priloga k znanstvenemu članku z naslovom: Cylindrospermopsin induced transcriptional responses in human hepatoma HepG2 cells.

Supplementary data 1: List of the selected genes with the deregulation data and decription

	12 h		24h				
gene symbol	Fold-change	p-value	Fold-change	p-value	Entrez Gene Name	NCBI Gene ID	molecular function (UniProtKB/Swiss-Prot)
Immediate-early response / signaling							
FOSB	**4.59**	2.34E-02	**8.58**	3.55E-03	FBJ murine osteosarcoma viral oncogene homolog B	2354	interacts with Jun proteins enhancing their DNA binding activity
FOS	**4.61**	1.70E-02	**4.59**	5.80E-03	FBJ murine osteosarcoma viral oncogene homolog	2353	forms a complex with the JUN/AP-1 transcription factor. forms a multimeric SMAD3/SMAD4/JUN/FOS complex e to regulate TGF-beta-mediated signaling
JUNB	**2.87**	5.95E-03	**4.71**	6.08E-04	jun B proto-oncogene	3726	transcription factor involved in regulating gene activity following the primary growth factor response
TGFB2	**2.27**	9.04E-02	**2.95**	1.66E-03	transforming growth factor. beta 2	7048	regulate the transcription of a subset of genes related to cell proliferation
JUN		NS	**2.58**	1.29E-03	jun proto-oncogene	3725	promotes activity of NR5A1 when phosphorylated by HIPK3 leading to increased steroidogenic gene expression upon cAMP signaling pathway stimulation
GDF15		NS	**2.46**	1.64E-03	growth differentiation factor 15	9518	role in regulating inflammatory and apoptotic pathways in injured tissues and during disease processes
NFKB1		NS	**2.03**	2.31E-03	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	4790	pleiotropic transcription factor which is present in almost all cell types and is involved in many biological processed such as inflammation. immunity. ferentiation. cell growth. tumorigenesis and apoptosis
GAB1	**1.84**	4.78E-02		NS	GRB2-associated binding protein 1	2549	role in intracellular signaling cascades triggered by activated receptor-type kinases. mediator of branching tubulogenesis and plays a central role in cellular growth response. transformation and apoptosis
TP53		NS		NS	tumor protein p53	7157	induces growth arrest or apoptosis. involved in cell cycle regulation as a trans-activator that acts to negatively regulate cell division by controlling a set of genes required for this process
MDM2		NS	1.33	3.29E-04	Mdm2 p53 binding protein homolog (mouse)	4193	E3 ubiquitin-protein ligase that mediates ubiquitination of p53/TP53. leading to its degradation by the proteasome
TGFA		NS	1.38	2.89E-02	transforming growth factor. alpha	7039	mitogenic polypeptide that is able to bind to the EGF receptor/EGFR and to act synergistically with TGF beta to promote anchorage-independent cell proliferation
TGFB1		NS	1.34	6.86E-03	transforming growth factor. beta 1	7040	Multifunctional protein that controls proliferation. differentiation and other functions in many cell types
MYC		NS	-1.28	4.99E-02	v-myc myelocytomatosis viral oncogene homolog (avian)	4609	participates in the regulation of gene transcription
ATM		NS		NS	ataxia telangiectasia mutated	472	DNA damage sensor. activates checkpoint signaling upon double strand breaks (DSBs). apoptosis and genotoxic stresses
JUND		NS		NS	jun D proto-oncogene	3727	Transcription factor binding AP-1 sites
NR1I2		NS		NS	nuclear receptor subfamily 1. group I. member 2	8856	activates the transcription of multiple genes involved in the metabolism and secretion of potentially harmful xenobiotics. drugs and endogenous compounds
NRG1		NS		NS	neuregulin 1	3084	signaling protein that mediates cell-cell interactions and plays critical roles in the growth and development of multiple organ systems
EGF		NS		NS	epidermal growth factor	1950	stimulates the growth of various epidermal and epithelial tissues
HGF	UND		UND		hepatocyte growth factor (hepapoietin A; scatter factor)	3082	potent mitogen for mature parenchymal hepatocyte cells. seems to be an hepatotrophic factor. and acts as growth factor
RAC1	UND		UND		ras-related C3 botulinum toxin substrate 1 (rho family. small GTP binding protein Rac1)	5879	binds to a variety of effector proteins to regulate cellular responses such as secretory processes. phagocytosis of apoptotic cells. epithelial cell polarization and membrane ruffles
cell cycle / proliferation							
GADD45B	**3.49**	2.01E-02	**4.95**	1.57E-03	growth arrest and DNA-damage-inducible. beta	4616	Involved in the regulation of growth and apoptosis
GADD45A	**1.68**	9.66E-03	**4.10**	1.52E-04	growth arrest and DNA-damage-inducible. alpha	1647	Involved in the regulation of growth and apoptosis
CDKN1A		NS	**3.71**	2.28E-03	cyclin-dependent kinase inhibitor 1A (p21. Cip1)	1026	binds to and inhibits the activity of cyclin-CDK2 or -CDK4 complexes. and thus functions as a regulator of cell cycle progression at G1
CDKN2B	**2.27**	1.12E-02		NS	cyclin-dependent kinase inhibitor 2B (p15. inhibits CDK4)	1030	interacts strongly with CDK4 and CDK6. potential effector of TGF-beta induced cell cycle arrest
HUS1	**1.72**	2.13E-02	**2.80**	1.19E-03	HUS1 checkpoint homolog (S. pombe)	3364	component of the 9-1-1 cell-cycle checkpoint response complex that plays a major role in DNA repair
CHEK1	1.38	2.05E-03	**2.42**	6.49E-03	checkpoint kinase 1	1111	required for checkpoint-mediated cell cycle arrest and activation of DNA repair in response to the presence of DNA damage or unreplicated DNA
CDK7	1.43	3.88E-02	**1.94**	1.99E-02	cyclin-dependent kinase 7	1022	involved in cell cycle control and in RNA polymerase II-mediated RNA transcription
CCNE2	**1.93**	4.66E-04	**1.61**	2.48E-03	cyclin E2	9134	essential for the control of the cell cycle at the late G1 and early S phase
E2F4		NS	**1.53**	5.80E-03	E2F transcription factor 4. p107/p130-binding	1874	transcription activator involved in cell cycle regulation or in DNA replication
PCNA	-1.38	3.79E-03	**-1.71**	2.56E-03	proliferating cell nuclear antigen	5111	processivity factor for DNA polymerase δ /RAD6-dependent DNA repair pathway and DNA synthesis
CDKN2C		NS	**-1.77**	3.86E-02	cyclin-dependent kinase inhibitor 2C (p18. inhibits CDK4)	1031	interacts strongly with CDK6. weakly with CDK4. inhibits cell growth and proliferation with a correlated dependence on endogenous retinoblastoma protein RB
CCNG1		NS	**-1.71**	7.01E-03	cyclin G1	900	associated with G2/M phase arrest in response to DNA damage

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UHRF1		NS	**-1.71**	2.27E-02	ubiquitin-like with PHD and ring finger domains 1	29128	a major role in the G1/S transition by regulating topoisomerase II alpha and retinoblastoma gene expression. and functions in the p53-dependent DNA damage checkpoint
TFDP1		NS	**-1.70**	4.66E-07	transcription factor Dp-1	7027	Can stimulate E2F-dependent transcription
RAD1		NS	**-1.69**	9.79E-04	RAD1 homolog (S. pombe)	5810	Component of the 9-1-1 cell-cycle checkpoint response complex that plays a major role in DNA repair
CDC27	1.28	4.33E-02	1.22	1.41E-02	cell division cycle 27 homolog (S. cerevisiae)	996	component of the anaphase promoting complex/cyclosome (APC/C). a cell cycle-regulated E3 ubiquitin ligase that controls progression through mitosis and the G1 phase of the cell cycle
RAD9A	-1.40	4.17E-02	-1.36	1.56E-02	RAD9 homolog A (S. pombe)	5883	Component of the 9-1-1 cell-cycle checkpoint response complex that plays a major role in DNA repair
CCND1	1.36	2.11E-03		NS	cyclin D1	595	regulatory component of the cyclin D1-CDK4 (DC) complex that phosphorylates and inhibits members of the retinoblastoma (RB) protein family including RB1 and regulates the cell-cycle during G(1)/S transition
CHEK2	-1.11	6.06E-03		NS	checkpoint kinase 2	11200	required for checkpoint-mediated cell cycle arrest. activation of DNA repair and apoptosis in response to the presence of DNA double-strand breaks
CCNC		NS	1.28	9.29E-03	cyclin C	892	a coactivator involved in regulated gene transcription of nearly all RNA polymerase II-dependent genes
RB1		NS	1.28	4.21E-04	retinoblastoma 1	5925	promotes G0-G1 transition. acts as a transcription repressor of E2F1 target genes
CDC2		NS	1.20	5.74E-03	cyclin-dependent kinase 1	983	promotes G2-M transition. and regulates G1 progress and G1-S transition via association with multiple interphase cyclins
CDKN1B		NS	-1.16	9.56E-03	cyclin-dependent kinase inhibitor 1B (p27. Kip1)	1027	important regulator of cell cycle progression. involved in G1 arrest. potent inhibitor of cyclin E- and cyclin A-CDK2 complexes
FOXM1		NS	-1.21	3.21E-02	forkhead box M1	2305	transcriptional factor regulating the expression of cell cycle genes essential for DNA replication and mitosis
CCNA2		NS		NS	cyclin A2	890	essential for the control of the cell cycle at the G1/S (start) and the G2/M (mitosis) transitions
CCNB1		NS		NS	cyclin B1	891	essential for the control of the cell cycle at the G2/M (mitosis) transition
CCND2		NS		NS	cyclin D2	894	regulatory component of the cyclin D2-CDK4 (DC) complex that phosphorylates and inhibits members of the retinoblastoma (RB) protein family including RB1 and regulates the cell-cycle during G(1)/S transition
CCNE1		NS		NS	cyclin E1	898	essential for the control of the cell cycle at the G1/S (start) transition
CDC20		NS		NS	cell division cycle 20 homolog (S. cerevisiae)	991	required for full ubiquitin ligase activity of the anaphase promoting complex/cyclosome (APC/C)
CDC25A		NS		NS	cell division cycle 25 homolog A (S. pombe)	993	functions as a dosage-dependent inducer of mitotic progression
CDC25C		NS		NS	cell division cycle 25 homolog C (S. pombe)	995	functions as a dosage-dependent inducer of mitotic progression
CDK2		NS		NS	cyclin-dependent kinase 2	1017	involved in the control of the cell cycle
CDK4		NS		NS	cyclin-dependent kinase 4	1019	component of cyclin D-CDK4 (DC) complexes that phosphorylate and inhibit members of the retinoblastoma (RB) protein family including RB1 and regulate the cell-cycle during G(1)/S transition
CDKN2A		NS		NS	cyclin-dependent kinase inhibitor 2A (melanoma. p16. inhibits CDK4)	1029	induces cell cycle arrest in G1 and G2 phases. acts as a tumor suppressor. binds to MDM2 and blocks its nucleocytoplasmic shuttling by sequestering it in the nucleolus
E2F1		NS		NS	E2F transcription factor 1	1869	transcription activator involved in cell cycle regulation or in DNA replication
MAD2L2		NS		NS	MAD2 mitotic arrest deficient-like 2 (yeast)	10459	component of the mitotic spindle assembly checkpoint that prevents the onset of anaphase until all chromosomes are properly aligned at the metaphase plate
PKMYT1		NS		NS	protein kinase. membrane associated tyrosine/threonine 1	9088	negative regulator of entry into mitosis (G2 to M transition)
TFDP2		NS		NS	transcription factor Dp-2 (E2F dimerization partner 2)	7029	can stimulate E2F-dependent transcription
ANAPC10		NS		NS	anaphase promoting complex subunit 10	10393	component of the anaphase promoting complex/cyclosome (APC/C). a cell cycle-regulated E3 ubiquitin ligase that controls progression through mitosis and the G1 phase of the cell cycle
DNA damage repair							
XPC	**3.11**	1.27E-03	**3.08**	6.35E-05	xeroderma pigmentosum. complementation group C	7508	involved in global genome nucleotide excision repair (GG-NER) by acting as damage sensing and DNA-binding factor component of the XPC complex
ERCC4	**2.30**	3.15E-05	**2.96**	6.67E-04	excision repair cross-complementing rodent repair deficiency. complementation group 4	2072	structure-specific DNA repair endonuclease responsible for the 5-prime incision during DNA repair. involved in homologous recombination that assists in removing interstrand cross-link
LIG4	**1.68**	2.00E-02	**1.95**	3.73E-04	ligase IV. DNA. ATP-dependent	3981	joins single-strand breaks in a double-stranded polydeoxynucleotide in an ATP-dependent reaction Involved in DNA non-homologous end joining (NHEJ) required for double-strand break repair and V(D)J recombination
MSH3		NS	**1.51**	1.57E-03	mutS homolog 3 (E. coli)	4437	component of the post-replicative DNA mismatch repair system (MMR)
XRCC2	**3.21**	3.39E-02		NS	X-ray repair complementing defective repair in Chinese hamster cells 2	7516	involved in the homologous recombination repair (HRR) pathway of double-stranded DNA. thought to repair chromosomal fragmentation. translocations and deletions
RAD51	**-2.31**	3.44E-04	**-3.42**	6.23E-05	RAD51 homolog (S. cerevisiae)	5888	participates in a common DNA damage response pathway associated with the activation of homologous recombination and double-strand break repair
MRE11A		NS	**-2.02**	1.08E-04	MRE11 meiotic recombination 11 homolog A (S. cerevisiae)	4361	component of the MRN complex. which plays a central role in double-strand break (DSB) repair. DNA recombination. maintenance of telomere integrity and meiosis
BRCA2		NS	**-1.66**	4.31E-02	breast cancer 2. early onset	675	transcription regulator
POLB	**-7.22**	8.31E-03	-1.14	3.21E-02	polymerase (DNA directed). beta	5423	polymerase that plays a key role in base-excision repair
NEIL1	-1.35	1.91E-02	-1.41	5.63E-03	nei endonuclease VIII-like 1 (E. coli)	79661	Involved in base excision repair
ERCC5	-1.37	1.47E-02	-1.44	2.46E-03	excision repair cross-complementing rodent repair deficiency. complementation group 5	2073	single-stranded structure-specific DNA endonuclease involved in DNA excision repair
ERCC2	1.18	1.45E-02	1.11	3.45E-02	excision repair cross-complementing rodent repair deficiency. complementation group 2	2068	ATP-dependent 5'-3' DNA helicase. component of the core-TFIIH basal transcription factor. involved in nucleotide excision repair (NER) of DNA by opening DNA around the damage. and in RNA transcription
APEX2	1.22	2.46E-02		NS	APEX nuclease (apurinic/apyrimidinic endonuclease) 2	27301	a weak apurinic/apyrimidinic (AP) endodeoxyribonuclease in the DNA base excision repair (BER) pathway of DNA lesions induced by oxidative and alkylating agents

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MLH1		NS	1.46	3.19E-05	mutL homolog 1. colon cancer. nonpolyposis type 2 (E. coli)	4292	component of the post-replicative DNA mismatch repair system (MMR)
TDG		NS	1.41	7.44E-03	thymine-DNA glycosylase	6996	corrects G/T mispairs to G/C pairs
RAD50		NS	1.19	2.65E-02	RAD50 homolog (S. cerevisiae)	10111	central role in double-strand break (DSB) repair. DNA recombination. maintenance of telomere integrity and meiosis
ERCC3		NS	1.16	4.16E-02	excision repair cross-complementing rodent repair deficiency. complementation group 3 (xeroderma pigmentosum group B complementing)	2071	ATP-dependent 3'-5' DNA helicase. acts byopening DNA either around the RNA transcription start site or the DNA damage
RAD21		NS	-1.10	1.04E-02	RAD21 homolog (S. pombe)	5885	repair of DNA double-strand breaks. chromatid cohesion during mitosis
OGG1		NS	-1.12	4.99E-02	8-oxoguanine DNA glycosylase	4968	incises DNA at 8-oxoG residues
MSH2		NS	-1.27	2.01E-02	mutS homolog 2. colon cancer. nonpolyposis type 1 (E. coli)	4436	component of the post-replicative DNA mismatch repair system (MMR)
APEX1		NS	-1.27	4.15E-03	APEX nuclease (multifunctional DNA repair enzyme) 1	328	functions as a apurinic/apyrimidinic (AP) endodeoxyribonuclease in the DNA base excision repair (BER) pathway of DNA lesions induced by oxidative and alkylating agents
DDB1		NS	-1.27	7.01E-03	damage-specific DNA binding protein 1. 127kDa	1642	functions in nucleotide-excision repair
NEIL2		NS	-1.35	2.05E-02	nei endonuclease VIII-like 2 (E. coli)	252969	involved in base excision repair
BRCA1		NS		NS	breast cancer 1. early onset	672	E3 ubiquitin-protein ligase that specifically mediates the formation of 'Lys-6'-linked polyubiquitin chains and plays a central role in DNA repair by facilitating cellular responses to DNA damage
ERCC1		NS		NS	excision repair cross-complementing rodent repair deficiency. complementation group 1 (includes overlapping antisense sequence)	2067	structure-specific DNA repair endonuclease responsible for the 5'-incision during DNA repair
LIG1		NS		NS	ligase I. DNA. ATP-dependent	3978	DNA **lig**ase that seals nicks in double-stranded DNA during DNA replication. DNA recombination and DNA repair
MGMT		NS		NS	O-6-methylguanine-DNA methyltransferase	4255	involved in the cellular defense against the biological effects of O6-methylguanine (O6-MeG) in DNA. repairs alkylated guanine in DNA by stoichiometrically transferring the alkyl group at the O-6 position to a cysteine residue in the enzyme
MUTYH		NS		NS	mutY homolog (E. coli)	4595	oxidative DNA damage repair
NTHL1		NS		NS	nth endonuclease III-like 1 (E. coli)	4913	apurinic and/or apyrimidinic endonuclease activity and a DNA N-glycosylase activity
POLD1		NS		NS	polymerase (DNA directed). delta 1. catalytic subunit 125kDa	5424	DNA synthesis (polymerase) and an exonucleolytic activity
PRKDC		NS		NS	protein kinase. DNA-activated. catalytic polypeptide	5591	sensor for DNA damage. involved in DNA nonhomologous end joining (NHEJ) required for double-strand break (DSB) repair and V(D)J recombination
SMUG1		NS		NS	single-strand-selective monofunctional uracil-DNA glycosylase 1	23583	recognizing base lesions in the genome and initiating base excision DNA repair
TREX1		NS		NS	three prime repair exonuclease 1	11277	major 3'->5' DNA exonuclease in human cells
UNG		NS		NS	uracil-DNA glycosylase	7374	Excises uracil residues from the DNA
XPA		NS		NS	xeroderma pigmentosum. complementation group A	7507	involved in DNA excision repair
XRCC4		NS		NS	X-ray repair complementing defective repair in Chinese hamster cells 4	7518	involved in DNA non-homologous end joining (NHEJ) required for double-strand break repair and V(D)J recombination

apoptosis / survival							
FAS	**2.73**	3.44E-04	**3.85**	5.45E-05	Fas (TNF receptor superfamily. member 6)	355	receptor for TNFSF6/FASLG. formes death-inducing signaling complex (DISC) with FADD. performs caspase-8 proteolytic activation
DIABLO	**1.69**	7.71E-04	**2.94**	7.49E-03	diablo. IAP-binding mitochondrial protein	56616	promotes apoptosis by activating caspases in the cytochrome c/Apaf-1/caspase-9 pathway
TIMP1	**1.97**	1.29E-02		NS	TIMP metallopeptidase inhibitor 1	7076	inhibitor of the matrix metalloproteinases (MMPs). a group of peptidases involved in degradation of the extracellular matrix. able to promote cell proliferation in a wide range of cell types
BCL2L1	**1.81**	1.06E-02	**2.43**	1.54E-04	BCL2-like 1	598	binding to it and preventing the release of the caspase activator. CYC1. from the mitochondrial membrane
TNF	**51.38**	1.86E-02	**27.11**	1.10E-02	tumor necrosis factor	7124	binds to TNFRSF1A/TNFR1 and TNFRSF1B/TNFBR. can induce cell death of certain tumor cell lines. involved in cellular responses to stimuli such as cytokines and stress and plays a key role in regulating the immune response to infection
TNFAIP3	**2.04**	4.36E-02	**3.41**	3.04E-04	tumor necrosis factor. alpha-induced protein 3	7128	inhibit NF-kappa B activation as well as TNF-mediated apoptosis. critical for limiting inflammation by terminating TNF-induced NF-kappa B responses
TNFRSF10A	1.40	4.04E-03	**1.95**	4.65E-04	tumor necrosis factor receptor superfamily. member 10a	8797	formes death-inducing signaling complex (DISC) with FADD. performs caspase-8 proteolytic activation
MCL1		NS	**1.90**	2.04E-04	myeloid cell leukemia sequence 1 (BCL2-related)	4170	regulation of apoptosis versus cell survival. and in the maintenance of viability
CASP9	**1.54**	5.13E-04	**1.82**	7.28E-05	caspase 9. apoptosis-related cysteine peptidase	842	involved in the activation cascade of caspases responsible for apoptosis execution
BAK1		NS	**1.81**	3.59E-04	BCL2-antagonist/killer 1	578	functions to induce apoptosis. interacts with and accelerates the opening of the mitochondrial voltage-dependent anion channel
TRADD	1.39	3.99E-03	**1.78**	6.54E-04	TNFRSF1A-associated via death domain	8717	a death domain containing adaptor molecule that interacts with TNFRSF1A/TNFR1 and mediates programmed cell death signaling and NF-kappaB activation
CASP3		NS	**1.75**	3.58E-02	caspase 3. apoptosis-related cysteine peptidase	836	involved in the activation cascade of caspases responsible for apoptosis execution
FOXO3	**1.87**	1.35E-02	**1.61**	1.63E-03	forkhead box O3	2309	transcriptional activator. triggers apoptosis in the absence of survival factors
DDIT3		NS	**1.56**	2.25E-03	DNA-damage-inducible transcript 3	1649	implicated in adipogenesis and erythropoiesis. is activated by endoplasmic reticulum stress. and promotes apoptosis
CASP8	1.40	2.98E-02	**1.57**	2.64E-03	caspase 8. apoptosis-related cysteine peptidase	841	involved in the activation cascade of caspases responsible for apoptosis execution
BCL2	**-3.25**	9.99E-03	**-3.12**	8.41E-04	B-cell CLL/lymphoma 2	596	suppresses apoptosis in a variety of cell systems. regulates cell death by controlling the mitochondrial membrane permeability

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TNFSF10		NS	**-2.97**	6.16E-04	tumor necrosis factor (ligand) superfamily. member 10	8743	binds to TNFRSF10A/TRAILR1. TNFRSF10B/TRAILR2. TNFRSF10C/TRAILR3. TNFRSF10D/TRAILR4 and induces apoptosis
BID	**-2.25**	3.68E-04	**-2.03**	3.65E-05	BH3 interacting domain death agonist	637	death agonist that heterodimerizes with either agonist BAX or antagonist BCL2. mediator of mitochondrial damage induced by caspase-8 (CASP8)
APAF1	-1.42	2.94E-03	**-1.73**	1.19E-03	apoptotic peptidase activating factor 1	317	mediates the cytochrome c-dependent autocatalytic activation of pro-caspase-9 (**Apaf-**3). leading to the activation of caspase-3 and apoptosis
CASP7	**-2.54**	2.01E-02		NS	caspase 7. apoptosis-related cysteine peptidase	840	involved in the activation cascade of caspases responsible for apoptosis execution
BAX		NS	1.42	9.98E-03	BCL2-associated X protein	581	accelerates programmed cell death by binding to. and antagonizing the apoptosis repressor BCL2 or its adenovirus homolog E1B 19k protein
BAG1		NS	1.37	3.18E-03	BCL2-associated athanogene	573	inhibits the pro-apoptotic function of PPP1R15A. has anti-apoptotic activity. increases the anti-cell death function of BCL2 induced by various stimuli
TRIB3	-1.40	4.68E-02	-1.41	2.01E-04	tribbles homolog 3 (Drosophila)	57761	negative regulator of NF-kappaB and can also sensitize cells to TNF- and TRAIL-induced apoptosis
BAD		NS		NS	BCL2-associated agonist of cell death	572	promotes cell death. successfully competes for the binding to Bcl-X(L). Bcl-2 and Bcl-W. thereby affecting the level of heterodimerization of these proteins with BAX
CASP2		NS		NS	caspase 2. apoptosis-related cysteine peptidase	835	involved in the activation cascade of caspases responsible for apoptosis execution
FADD		NS		NS	Fas (TNFRSF6)-associated via death domain	8772	apoptotic adaptor molecule that recruits caspase-8 or caspase-10 to the activated Fas (CD95) or TNFR-1 receptors
detoxificaton response							
CAT		NS	**4.59**	1.59E-02	catalase	847	serves to protect cells from the toxic effects of hydrogen peroxide
ALDH1A2	UND		**5.53**	1.36E-02	aldehyde dehydrogenase 1 family. member A2	8854	catalyzes the synthesis of retinoic acid (RA) from retinaldehyde
CYP1A1	**5.96**	3.66E-04	**2.98**	2.61E-02	cytochrome P450. family 1. subfamily A. polypeptide 1	1543	involved in an NADPH-dependent electron transport pathway. It oxidizes a variety of structurally unrelated compounds. including steroids. fatty acids. and xenobiotics
CYP1B1	**3.47**	3.27E-03		NS	cytochrome P450. family 1. subfamily B. polypeptide 1	1545	involved in an NADPH-dependent electron transport pathway. It oxidizes a variety of structurally unrelated compounds. including steroids. fatty acids. and xenobiotics
UGT1A6	**6.81**	9.63E-03		NS	UDP glucuronosyltransferase 1 family. polypeptide A6	54578	an enzyme of the glucuronidation pathway that transforms small lipophilic molecules. such as steroids. bilirubin. hormones. and drugs. into water-soluble. excretable metabolites
TXNRD1	1.44	1.96E-02	**2.09**	1.02E-03	thioredoxin reductase 1	7296	reduces thioredoxins as well as other substrates. and plays a role in selenium metabolism and protection against oxidative stress
NAT1	1.34	4.84E-03	**1.94**	4.84E-04	N-acetyltransferase 1 (arylamine N-acetyltransferase)	9	N- or O-acetylation of various arylamine and heterocyclic amine substrates
GCLC	1.35	2.21E-03	**1.93**	2.44E-03	glutamate-cysteine ligase. catalytic subunit	2729	the first rate limiting enzyme of glutathione synthesis
CES2	1.39	1.58E-02	**1.77**	1.84E-02	carboxylesterase 2	8824	involved in the detoxification of xenobiotics and in the activation of ester and amide prodrugs
GSTM3	**1.52**	1.67E-02	**1.69**	8.59E-05	glutathione S-transferase mu 3 (brain)	2947	conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles
UGT1A1	**1.67**	3.28E-02		NS	UDP glucuronosyltransferase 1 family. polypeptide A1	54658	an enzyme of the glucuronidation pathway that transforms small lipophilic molecules. such as steroids. bilirubin. hormones. and drugs. into water-soluble. excretable metabolites
CYP2A6	**-3.74**	1.17E-02		NS	cytochrome P450. family 2. subfamily A. polypeptide 6	1548	hydroxylates coumarin. and also metabolizes nicotine. aflatoxin B1. nitrosamines. and some pharmaceuticals
CYP2A13		NS	**-3.72**	4.00E-02	cytochrome P450. family 2. subfamily A. polypeptide 6	1553	its endogenous substrate has not been determined. it is known to metabolize 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. a major nitrosamine specific to tobacco
CYP3A43		NS	**-2.48**	1.52E-02	cytochrome P450. family 3. subfamily A. polypeptide 43	64816	xhibits low testosterone 6-beta-hydroxylase activity
CYP3A7		NS	**-2.36**	4.74E-04	cytochrome P450. family 3. subfamily A. polypeptide 7	1551	involved in an NADPH-dependent electron transport pathway. It oxidizes a variety of structurally unrelated compounds. including steroids. fatty acids. and xenobiotics
GSTM2	-1.40	5.18E-03	**-2.16**	2.78E-04	glutathione S-transferase mu 2 (muscle)	2946	conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles
CYP2F1	**-2.43**	1.10E-03	**-2.03**	3.41E-03	cytochrome P450. family 2. subfamily F. polypeptide 1	1572	dehydrogenates 3-methylindole. an endogenous toxin derived from the fermentation of tryptophan. as well as xenobiotic substrates such as naphthalene and ethoxycoumarin
GSTA2		NS	**-1.76**	4.24E-03	glutathione S-transferase alpha 2	2939	conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles
CES1	-1.49	2.08E-02	**-1.74**	3.95E-03	carboxylesterase 1	1066	involved in the detoxification of xenobiotics and in the activation of ester and amide prodrugs
GNMT		NS	**-1.63**	3.76E-02	glycine N-methyltransferase	27232	catalyzes the synthesis of N-methylglycine (sarcosine) from glycine using S-adenosylmethionine (AdoMet) as the methyl donor
SULT1A1		NS	**-1.58**	1.10E-03	sulfotransferase family. cytosolic. 1A. phenol-preferring. member 1	6817	catalyze the sulfate conjugation of many hormones. neurotransmitters. drugs. and xenobiotic compounds
GSR	-1.37	1.43E-04	-1.29	3.56E-03	glutathione reductase	2936	maintains high levels of reduced glutathione in the cytosol
SULT1E1		NS	1.45	2.52E-02	sulfotransferase family 1E. estrogen-preferring. member 1	6783	transfers a sulfo moiety to and from estrone
GSTA4		NS	-1.17	1.53E-02	glutathione S-transferase alpha 4	2941	conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles
CYP2E1		NS	-1.26	4.09E-02	cytochrome P450. family 2. subfamily E. polypeptide 1	1571	metabolizes several precarcinogens. drugs. and solvents to reactive metabolites. Inactivates a number of drugs and xenobiotics and also bioactivates many xenobiotic substrates to their hepatotoxic or carcinogenic forms
CYP2D6		NS	-1.35	3.81E-02	cytochrome P450. family 2. subfamily D. polypeptide 6	1565	responsible for the metabolism of many drugs and environmental chemicals that it oxidizes. involved in the metabolism of drugs such as antiarrhythmics. adrenoceptor antagonists. and tricyclic antidepressants
GPX3		NS	-1.49	4.13E-02	glutathione peroxidase 3 (plasma)	2878	protects cells and enzymes from oxidative damage. by catalyzing the reduction of hydrogen peroxide. lipid peroxides and organic hydroperoxide. by glutathione
CYP3A5	1.31	3.87E-03		NS	cytochrome P450. family 3. subfamily A. polypeptide 5	1577	involved in an NADPH-dependent electron transport pathway. It oxidizes a variety of structurally unrelated compounds. including steroids. fatty acids. and xenobiotics
GPX4	-1.21	1.78E-02		NS	glutathione peroxidase 4 (phospholipid hydroperoxidase)	2879	protects cells and enzymes from oxidative damage. by catalyzing the reduction of hydrogen peroxide. lipid peroxides and organic hydroperoxide. by glutathione
ALDH1A1	-1.17	3.92E-03		NS	aldehyde dehydrogenase 1 family. member A1	216	binds free retinal and cellular retinol-binding protein-bound retinal. Can convert/oxidize retinaldehyde to retinoic acid (By similarity)
NQO1	-1.37	3.82E-03		NS	NAD(P)H dehydrogenase. quinone 1	1728	NAD(P)H dehydrogenase (quinone) activity

se nadaljuje

nadaljevanje: Priloga k znanstvenemu članku z naslovom: Cylindrospermopsin induced transcriptional responses in human hepatoma HepG2 cells.

CYP1A2		NS		NS	cytochrome P450. family 1. subfamily A. polypeptide 2	1544	involved in an NADPH-dependent electron transport pathway. It oxidizes a variety of structurally unrelated compounds. including steroids. fatty acids. and xenobiotics
CYP2A7		NS		NS	cytochrome P450. family 2. subfamily A. polypeptide 6	1549	involved in an NADPH-dependent electron transport pathway. It oxidizes a variety of structurally unrelated compounds. including steroids. fatty acids. and xenobiotics
CYP2B6		NS		NS	cytochrome P450. family 2. subfamily B. polypeptide 6	1555	involved in an NADPH-dependent electron transport pathway. It oxidizes a variety of structurally unrelated compounds. including steroids. fatty acids. and xenobiotics
CYP2C18		NS		NS	cytochrome P450. family 2. subfamily C. polypeptide 18	1562	involved in an NADPH-dependent electron transport pathway. It oxidizes a variety of structurally unrelated compounds. including steroids. fatty acids. and xenobiotics
CYP2C19		NS		NS	cytochrome P450. family 2. subfamily C. polypeptide 19	1557	responsible for the metabolism of a number of therapeutic agents such as the anticonvulsant drug S-mephenytoin. omeprazole. proguanil. certain barbiturates. diazepam. propranolol. citalopram and imipramine
CYP2C8		NS		NS	cytochrome P450. family 2. subfamily C. polypeptide 8	1558	involved in an NADPH-dependent electron transport pathway. It oxidizes a variety of structurally unrelated compounds. including steroids. fatty acids. and xenobiotics
CYP2C9		NS		NS	cytochrome P450. family 2. subfamily C. polypeptide 9	1559	involved in an NADPH-dependent electron transport pathway. It oxidizes a variety of structurally unrelated compounds. including steroids. fatty acids. and xenobiotics
CYP2S1		NS		NS	cytochrome P450. family 2. subfamily S. polypeptide 1	29785	potential importance for extrahepatic xenobiotic metabolis
CYP3A4		NS		NS	cytochrome P450. family 3. subfamily A. polypeptide 4	1576	involved in an NADPH-dependent electron transport pathway. It oxidizes a variety of structurally unrelated compounds. including steroids. fatty acids. and xenobiotics
DPYD		NS		NS	dihydropyrimidine dehydrogenase	1806	involved in pyrimidine base degradation. atalyzes the reduction of uracil and thymine. also involved the degradation of the chemotherapeutic drug 5-fluorouracil
EPHX1		NS		NS	epoxide hydrolase 1. microsomal (xenobiotic)	2052	catalyzes the hydrolysis of arene and aliphatic epoxides to less reactive and more water soluble dihydrodiols by the trans addition of water
FMO4		NS		NS	flavin containing monooxygenase 4	2329	involved in the oxidative metabolism of a variety of xenobiotics such as drugs and pesticides
GGT1		NS		NS	gamma-glutamyltransferase 1	2678	initiates extracellular glutathione (GSH) breakdown. provides cells with a local cysteine supply and contributes to maintain intracelular GSH level
GSS		NS		NS	glutathione synthetase	2937	catalyzes the second step of glutathione biosynthesis
GSTA5		NS		NS	glutathione S-transferase alpha 5	221357	conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles
GSTK1		NS		NS	glutathione S-transferase kappa 1	373156	conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles
GSTM1		NS		NS	glutathione S-transferase mu 1	2944	conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles
GSTT1		NS		NS	glutathione S-transferase theta 1	2952	conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles
MAOB		NS		NS	monoamine oxidase B	4129	catalyzes the oxidative deamination of biogenic and xenobiotic amines
NAT2		NS		NS	N-acetyltransferase 2 (arylamine N-acetyltransferase)	10	N- or O-acetylation of various arylamine and heterocyclic amine substrates
NOS2		NS		NS	nitric oxide synthase 2. inducible	18126	Produces nitric oxide (NO)
POR		NS		NS	P450 (cytochrome) oxidoreductase	5447	transfer from NADP to cytochrome P450 in microsomes
SOD1		NS		NS	superoxide dismutase 1. soluble	6647	responsible for destroying free superoxide radicals
TYMS		NS		NS	thymidylate synthetase	7298	catalyzes the methylation of deoxyuridylate to deoxythymidylate using 5.10-methylenetetrahydrofolate (methylene-THF) as a cofactor
UGT1A4		NS		NS	UDP glucuronosyltransferase 1 family. polypeptide A4	54657	an enzyme of the glucuronidation pathway that transforms small lipophilic molecules. such as steroids. bilirubin. hormones. and drugs. into water-soluble. excretable metabolites
UGT1A7		NS		NS	UDP glucuronosyltransferase 1 family. polypeptide A9	54577	an enzyme of the glucuronidation pathway that transforms small lipophilic molecules. such as steroids. bilirubin. hormones. and drugs. into water-soluble. excretable metabolites
NOX4	UND			NS	NADPH oxidase 4	50507	superoxide-generating NADPH oxidase
CYP4B1	UND		UND		cytochrome P450. family 4. subfamily B. polypeptide 1	1580	involved in an NADPH-dependent electron transport pathway. It oxidizes a variety of structurally unrelated compounds. including steroids. fatty acids. and xenobiotics
FMO3	UND		UND		flavin containing monooxygenase 3	2328	involved in the oxidative metabolism of a variety of xenobiotics such as drugs and pesticides
GSTM5	UND		UND		glutathione S-transferase mu 5	2949	conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles
NNMT	UND		UND		nicotinamide N-methyltransferase	4837	N-methylation of nicotinamide and other pyridines
XDH	UND		UND		xanthine dehydrogenase	7498	involved in the oxidative metabolism of purines
other							
HDAC1		NS		NS	histone deacetylase 1	3065	deacetylation of lysine residues on the N-terminal part of the core histones (H2A. H2B. H3 and H4). important role in transcriptional regulation. cell cycle progression and developmental events
ABCC5		NS		NS	ATP-binding cassette. sub-family C (CFTR/MRP). member 5	10057	acts as a multispecific organic anion pump which can transport nucleotide analogs
TUBA1A		NS		NS	tubulin. alpha 1a	7846	major constituent of microtubules. cytoskeleton
PTGS2		NS		NS	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	5743	formation of prostaglandins from arachidonate
SAA1		NS		NS	serum amyloid A1	6288	a major acute phase protein that is highly expressed in response to inflammation and tissue injury
GAPDH	-1.29	1.70E-02		NS	glyceraldehyde-3-phosphate dehydrogenase	2597	playing a role in glycolysis and nuclear functions

Genes significantly deregulated by CYN exposure for more than 1.5-fold are indicated by bold fond.

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