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Genotoxic effects of the cyanobacterial pentapeptide nodularin in HepG2 cells

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ABSTRACT

The cyanobacterial pentapeptide nodularin (NOD), mainly produced by genus *Nodularia*, is a potent inhibitor of protein phosphatases PP1 and PP2A, and causes animal mortality. The few studies available indicate that NOD is a potential non-genotoxic carcinogen. In the present study we evaluated NOD (0.01, 0.1 and 1 μg/ml) genotoxic activity in human hepatoma (HepG2) cells with the comet, γH2AX and cytokinesis block micronucleus cytome assays. In addition, induction of oxidative stress was studied. Moreover changes in the expression of selected genes from the P53 pathway, involved in the response to DNA damage (*P53*, *GADD45α*, *CDKN1A, MDM2*), apoptosis (*BAX, BCL2*) and oxidative stress (*GPX1*, *GSR, GCLC, CAT, SOD1*) were determined using qPCR. Non-cytotoxic concentrations induced time and dose dependant increase in reactive oxygen species (ROS) production and substantially increased the formation of oxidative DNA damage. In addition, elevated formation of micronuclei was detected. For the first time it has been shown that NOD deregulated the mRNA level of DNA damage (*CDKN1A, GADD45α*) and oxidative stress (*GPX1, GSR, GCLC, CAT* and *SOD1*) responsive genes and anti-apoptotic gene *BCL2*. Our results provide new evidence that NOD genotoxic effects are mediated through ROS production, already at low environmentally relevant concentrations.

1. Introduction

The cyclic pentapeptide nodularins (NODs) are mainly produced by *Nodularia spumigena*, which is spread across the world, preferably inhabiting brackish and estuarine environments (Pearson et al., 2010; Svircev et al., 2015; Buratti et al., 2017). Blooms of *N. spumigena* and occurrence of NODs have been reported worldwide from Australia (Heresztyn and Nicholson, 1997), New Zealand (Carmichael et al., 1988), from North America (Loftin et al., 2016; Beversdorf et al., 2017) and European countries, Spain (Beltran et al., 2012), Greece (Kaloudis et al., 2013), Turkey (Rodriguez et al., 2017), Serbia (Drobac et al., 2016), Germany (Greer et al., 2016; Rodriguez et al., 2017), and regularly during summer months they occur every year in the Baltic Sea (Barda et al., 2015; Laamanen et al., 2001; Mazur-Marzec and Pliński, 2009). Nine naturally occurring isoforms of NODs have been identified so far, from which nodularin-R (NOD) (Fig. 1) seems to be the main component of environmental samples, with other variants occurring rarely and in negligible concentrations (Mazur-Marzec et al., 2006; Sivonen et al., 1989; Buratti et al., 2017). NODs are similar in structure to microcystins (MCs). In addition to the Mdhb (N-methyldehydrobutyric acid) and 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, NODs contain also two D-amino acids, D-glutamic acid (D-Glu) and D-erythro-B-methylaspartic acid (D-MeAsp), and L-arginine at position 2. Structurally NODs have smaller ring structure than MCs and are therefore probably more easily taken into the hepatocytes than MCs.

The molecular mechanisms and the effects of MCs are fairly well investigated, while on the other hand the mechanisms of action and the effects of NODs are less known. The primary target organ of NODs is the liver and the principal mechanism of their toxicity is the specific inhibition of eukaryotic protein serine/threonine phosphatases 1, 2A and

Abbreviations: BaP, benzo[a]pyrene; BNC, binucleated cells; CBMN, cytokinesis block micronucleus assay; DCFH-DA, 2′,7′-dichlorfluorescein-diacetate; DSBs, double strand breaks; ET, etoposide; Fpg, formamidopyrimidine glycosylase; MC, microcystin; MNed, micronucleated; MNi, micronuclei; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NDI, nuclear division index; NBUD, nuclear bud; NOD, Nodularin; NPB, nucleoplasmic bridge; ROS, reactive oxygen species; SSBs, single strand breaks; PI, propidium iodide; TBHP, tert-Butyl hydroperoxide

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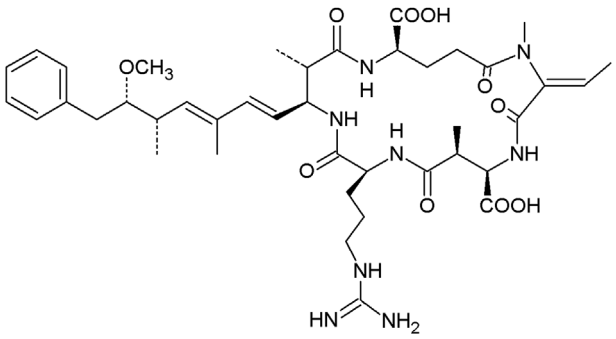

Fig. 1. Structure of nodularin.

3 (PP1, PP2A and PP3) (Eriksson et al., 1990; Runnegar et al., 1987). In contrast to MCs, NODs do not bind covalently to protein phosphatases (Bagu et al., 1997). The consequence of PP inhibition is hyperphosphorylation of cellular proteins, which leads to the 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; Hjørnevik et al., 2012)).

Similarly as MCs also NODs do not readily penetrate through the cell membrane by diffusion, but require multi-specific organic anion transporting polypeptides (Oatps) for uptake (Faltermann et al., 2016a). When inside the cell they produce intracellular reactive oxygen species (ROS) and thereby induce oxidative stress, causing lipid peroxidation as well as protein and DNA damage (for review see: (Chen et al., 2013; Zegura et al., 2011)). NODs are considered to act predominantly as a tumor promoters through the protein phosphatase inhibition and have much stronger tumor-promoting activity than MCs. They can even exert tumor-initiating activity and there is evidence that they are genotoxic *in vitro* (Bouaïcha et al., 2005; Lankoff et al. 2006, 2008; Maatouk et al., 2004). However, the mechanisms by which these toxins damage DNA and induce liver cancer are not well understood.

NODs have been implicated in several cases of animal mortalities, causing massive liver hemorrhage (Pearson et al., 2010), but fortunately no cases of human intoxications directly attributed to NODs have been reported so far. The concentrations of NOD determined in drinking water are within 1 μg/l (Yen et al., 2011; Buratti et al., 2017); however, in the environment the concentrations can range from μg/l and up to several mg/l (Mazur-Marzec et al., 2013; Balest et al., 2016; Buratti et al., 2017). Nevertheless the toxins bioaccumulate and have been found in tissues of clams, shrimps and fish - all used for consumption (Barda et al., 2015; Pearson et al., 2010; Sotton et al., 2014; Stewart et al., 2012; Van Buynder et al., 2001; Drobac et al., 2016; Foss et al., 2017). For example the concentrations found in aquatic organisms of the Baltic Sea can reach 0.91 μg/g dry weight in fish muscle and 2.5 μg/g in mussels (Mazur-Marzec et al., 2013). The toxins are not easily degraded by light, temperature and microwaves (Van Buynder et al., 2001). As they are persistent within living cells they can be transferred up the trophic levels (Karjalainen et al., 2003; Bownik, 2016), which means that chronic exposure of humans to low doses of NODs is possible. This is of great concern as NODs are potential human carcinogens although the details of their hepatotoxicity and carcinogenicity have not been fully elucidated. Therefore, the information on their genotoxicity and potential carcinogenicity is urgently needed.

The aim of the present study was to investigate NOD-induced oxidative stress and its genotoxic activity in the human hepatoma cell line, HepG2, by analysing intracellular reactive oxygen species production, DNA single- and double-strand break formation, influence on genomic instability by analysing the formation of micronuclei (MNi), nuclear buds (NBUD), and nucleoplasmic bridges (NPB) and NOD-mediated modulation of the expression of selected DNA damage-responsive genes and genes involved in programmed cell death and oxidative stress.

2. Materials and methods

2.1. Chemicals

Nodularin (NOD) was purchased from Enzo Life Sciences GmbH, Lausen, Switzerland. A 1 mg/ml stock solution of NOD was prepared in 50% methanol. William's medium E, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), glycogen, ethylenediaminetetraacetic acid (EDTA), tert-butyl hydroperoxide (TBHP), benzo[a]pyrene (BaP), and dimethylsulphoxide (DMSO) were 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. 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. Mouse monoclonal IgG1, Anti-phospho-Histone H2A.X (Ser139), FITC conjugate, were from Milipore, Billerica, Massachusetts, USA. cDNA High Capacity Archive Kit, TaqMan Universal PCR Master Mix and the Taqman Gene Expression Assays were from Applied Biosystems, New Jersey, USA. 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

HepG2 cell line was 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 humidified atmosphere in William's medium E supplemented with 15% foetal bovine serum, 100 U/ml penicillin/streptomycin and 2 mM L-glutamine.

2.3. Cell viability – MTT assay

Cell viability after the exposure to NOD was determined with the MTT assay according to Mosmann (1983) as described in Straser et al. (2011). Cells were treated with NOD (0, 0.01, 0.05, 0.1, 0.5, 1, 5 and 10 μg/ml) for 24 and 72 h.

2.4. Intracellular reactive oxygen species (ROS) formation

For measurement of intracellular ROS formation with a fluorescent probe, DCFH-DA, the cells were seeded (2.5×10^4/well) into black 96-well tissue culture treated microtiter plates in five replicates for 24 h at 37 °C in 5% CO_2. The procedure was conducted as described in Straser et al. (2013a). A vehicle control (1 × PBS containing 0.05% methanol) and a positive control TBHP (0.5 mM) were included in each experiment. For kinetic analyses the fluorescence intensity was measured every 30 min using a microplate reading spectrofluorimeter (Tecan, Genios) at the excitation and emission wavelength of 485 and 530 nm, respectively.

2.5. DSB detection – H2AX foci analysis

HepG2 cells were seeded on T-25 flasks (800 000 cells/plate), left to attach overnight and subsequently exposed to NOD (0, 0.01, 0.1 and 1 μg/ml) for 24 (short time exposure) and 72 h (long time exposure). In each experiment a vehicle control (cell growth medium containing 0.05% methanol) and a positive control etoposide (1 μg/ml ET, 24 h) were included. The procedure for cell fixations was conducted as

described in Straser et al. (2013b). Fixed cells were labelled in 1 × PBS containing 2000-fold diluted anti-γH2AX antibodies and 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. Ten thousand events were recorded in each sample. Independent experiments were repeated three times. For the 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 available on the website: http://research.stowers-institute.org/efg/ScientificSoftware/Utility/FCSExtract/index.htm.

2.6. Detection of SSB and oxidized DNA bases - comet assay

Cells were seeded (60 000 cells/well) into 12-well microtiter plates (Corning Costar Corporation, Corning, NY, USA), incubated for 24 h at 37 °C in 5% CO_2 and subsequently exposed to 0, 0.01, 0.1 and 1 μg/ml NOD for 4, 12 and 24 h. In each experiment, a vehicle control (cell growth medium containing 0.05% methanol) and a positive control (30 μM BaP) were included. The comet assay was conducted according to Singh et al., (1988) as described in Zegura et al. (2004). The levels of oxidative damage were examined with the modified comet assay according to Collins et al., (1993), as described in Zegura et al. (2003). The experiments were performed in three independent repetitions for each of the treatment conditions. From each treatment group images of 50 randomly selected nuclei per experimental point were analyzed with image analysis software Comet Assay IV (Perceptive Instruments, UK) and DNA damage was expressed as % of tail DNA.

2.7. Cytokinesis block micronucleus assay (CBMN)

Cells were seeded on T-25 flasks (Corning Costar Corporation, Corning, NY, USA) (700 000 cells/flask), incubated for 24 h at 37 °C and 5% CO_2 and subsequently exposed to 0.01, 0.1 and 1 μg/ml NOD for 24 h. ET and BaP (1 μg/ml and 10 μM, respectively) were used as positive controls, while 0.05% methanol in growth medium was a vehicle control. The CBMN assay was conducted as described in Straser et al. (2011). Micronuclei (MNi), nuclear bridges (NPBs) and nuclear buds (NBUDs) were evaluated in 1000 binucleated cells (BNC) per experimental point at 400 × magnification (Fig. 2), and were scored according to criteria published by Fenech, (2006). 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)]/1000, where M1–M4 represent the number of cells with one to four nuclei, respectively. The experiments were repeated three times, independently.

2.8. Gene expression

Cells were seeded on T-25 flasks (Corning Costar Corporation, Corning, NY, USA) (10^6 cells/flask), incubated for 24 h at 37 °C and 5% CO_2 and subsequently exposed to NOD (0.01, 0.1 and 1 μg/ml) 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. Total RNA was isolated with TRIzol® reagent, quantified on a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA) and reverse transcribed (1 μg) to cDNA using the High Capacity cDNA Reverse Transcription Kit. All procedures were performed according to the manufacturer's instructions with minor modifications described in Straser et al. (2011). Gene expression was quantified using real-time quantitative PCR (ABI 7900 HT Sequence Detection System, Applied Biosystems, USA). TaqMan Universal PCR Master Mix and Taqman Gene Expression Assays were used (derived from Applied Biosystems; New Jersey, USA): *P53* (tumor protein p53), Hs00153349_m1; *MDM2* (Mdm2, 'transformed 3T3 cell double minute 2′, p53 binding protein gene), Hs00234753_m1; *CDKN1A* ('cyclin-dependent kinase inhibitor 1A′), Hs00355782_m1; *GADD45A* (‘growth arrest and DNA damage-inducible gene, alpha'), Hs00169255_m1; *BCL2* (B-cell CLL/lymphoma 2), Hs00608023_m1; *BAX* (BCL2 associated X protein), Hs99999001_m1; *GCLC* (glutamate-cysteine ligase, catalytic subunit), Hs00155249 m1; *GSR* (glutathione reductase), Hs00167317 m1; *GPX1* (glutathione peroxidase 1), Hs01028922 g1; *CAT* (catalase), Hs00937387 m1; *SOD1* (superoxide dismutase 1, soluble), Hs00166575 m1.

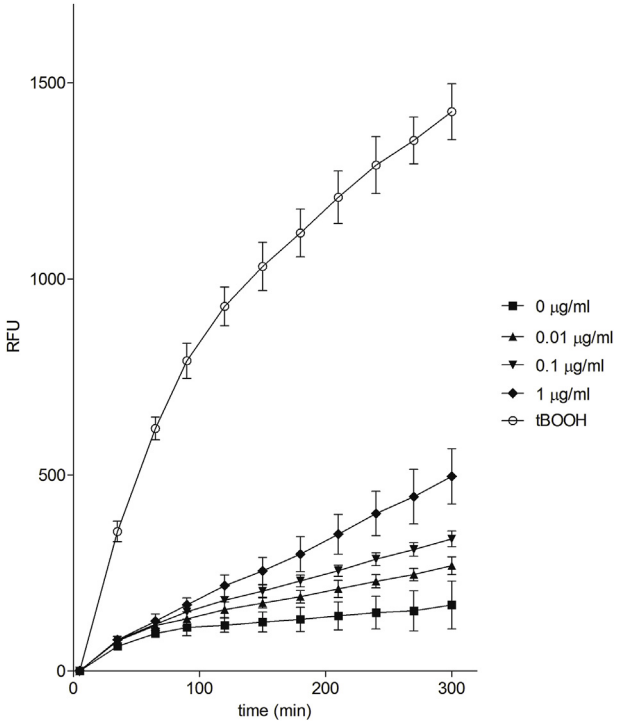

Fig. 2. NOD-induced increase of DCF fluorescence in HepG2. The HepG2 cells were pretreated with DCFH-DA (20 μM) for 30 min, washed and then exposed to different concentrations of NOD (0.01, 0.1 and 1 μg/ml). NOD concentrations that induced significant increase in DCF-fluorescence compared to the vehicle 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 and is expressed as relative fluorescence units (RFU).

Amplification of GAPDH probe (Human Endogenous Controls, Cat. No.:4310884E) was performed as an internal control. The following conditions for PCR were applied: 50 °C (2 min), 95 °C (10 min) and 40 cycles at 95 °C (15 s) and 60 °C (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.

2.9. Statistical analysis

The statistical analyses were performed with GraphPad Prism 5 software 5 (GraphPad Software, San Diego, CA, USA). One-way analysis of variance (ANOVA) was used to analyze the differences between treatments within each experiment in the comet assay. Dunnett's test was used for multiple comparisons versus the control. The significance of CBMN assay parameters was tested using chi-square test. $P < 0.05$ (*), 0.01 (**), 0.001 (***) were considered as statistically significant. In the MTT test, ROS formation, and gene expression the 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. For the γH2AX foci analysis the statistical

significance between treated groups and the vehicle control was determined with a linear mixed-effects model using the statistical program R (R Core Team, 2013) and its packages reshape (Wickham, 2007), ggplot2 (Wickham, 2009) and nlme (Pinheiro et al., 2013).

3. Results

3.1. NOD influence on viability of HepG2 cells

After 24 and 72 h of exposure NOD (0.01, 0.1 and 1 μg/ml) did not statistically significant affect the viability of HepG2 cells (data not shown); therefore, the concentrations of up to 1 μg/ml were used for further experiments.

3.2. Induction of reactive oxygen species (ROS)

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. 2), which steadily increased with prolonged incubation time. After 5 h incubation, we determined 2.5-fold and 7.3-fold increase in the fluorescence intensity at 1 μg/ml NOD and positive control (0.5 mM TBHP), respectively, compared to vehicle control. DCF fluorescence intensity slightly increased also in control cells, suggesting the formation of ROS in non-treated HepG2 cells under the experimental conditions used.

3.3. Induction of DNA double-strand breaks

Induction of DSBs by NOD was assesses for the first time by measuring the fluorescent signals of individual cells, indirectly through the detection of γH2AX foci analyzed by flow cytometry. The results showed that NOD did not induce statistically significant increase of DNA double strand breaks after 24 (short term exposure; Fig. 3a) and 72 h (long term exposure; Fig. 3b).

3.4. Induction of DNA single-strand breaks

The formation of SSBs in HepG2 cells after NOD exposure was determined with the comet assay. No significant increase in the amount of DNA SSBs was observed after 4 h of exposure to NOD (Fig. 4). There was a slight increase in DNA damage after 12 h, while after 24 h of exposure statistically significant dose dependent increase in DNA damage was detected at 0.1 and 1 μg/ml.

3.5. Induction of oxidized purines

Oxidized purines were detected with the purified DNA damage specific enzyme, formamidopyrimidine glycosylase (Fpg) (Collins, 2009). After 4 h of exposure NOD significantly increased the amount of Fpg sensitive sites at all concentrations tested, while no DNA damage was detected without Fpg digestion (Fig. 5). After 12 and 24 h of exposure the amount of Fpg-sensitive sites decreased; however, it was still statistically significant at concentrations 0.1 and 1 μg/ml. There was also some DNA damage without Fpg digestion at these time points and concentrations detected; however, it was less pronounced than after the enzyme digestion. These results indicate that DNA damage caused by NOD is probably the consequence of oxidative stress.

3.6. Induction of micronuclei, nucleoplasmic bridges and nuclear buds

NOD genotoxic potential in HepG2 cells was further evaluated using the CBMN cytome assay. Following 24 h exposure NOD induced dose dependent increase in the frequencies of MNi, while there were no differences in the number of NBUDs and NBPs in BNCs compared to vehicle control (Fig. 6). Statistically significant increases in the frequencies of MNi (Fig. 6a) were detected at 1 μg/ml NOD ($p < 0.05$)

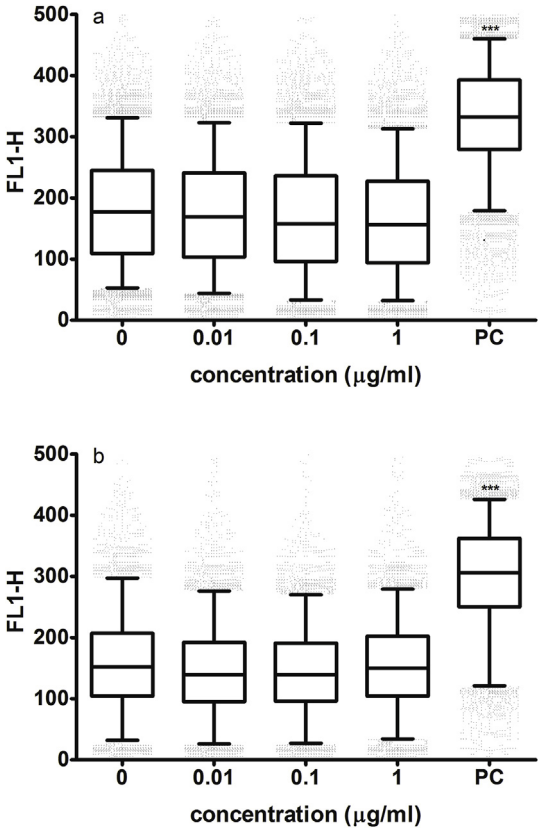

Fig. 3. Induction of double strand breaks (DSB) by NOD. HepG2 cells were incubated for (a) 24 and (b) 72 h with NOD (0.01, 0.1 and 1 μg/ml) and the presence of DSBs was analyzed by flow cytometry, indirectly through the detection of γH2A.X foci. In each experiment a vehicle control (0, 0.05% methanol) and a positive control (PC, 1 μg/mL etoposide, 24 h) were included. 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 independently. Significant difference between treated cells and the vehicle control (0) is indicated by * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

and were about 2.1-fold higher than in the control cells. At tested concentrations the cell division (NDI) was not influenced by NOD (Fig. 6d). Positive controls, BaP (10 μM) and ET (1 μg/ml) induced statistically significant increased frequencies of MNi (2.1-fold and 5.1-fold, respectively), but not NBUDs, while ET increased also the number of NPBs (15.4-fold).

3.7. Effect of NOD on the mRNA expression of DNA damage, oxidative stress and apoptosis responsive genes

The expressions of selected DNA damage responsive genes, genes involved in oxidative stress and apoptosis were analyzed after 4, 12 and 24 h exposure of HepG2 cells to NOD (0.01, 0.1 and 1 μg/ml) by quantitative real-time PCR (Table 1). NOD did not induce any changes in the mRNA expression of *TP53* at tested concentrations and exposure times. It caused slight though significant down-regulation of p53 downstream regulated genes, *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 all concentrations tested. There was only slight though statistically significant 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 pro-apoptotic gene *BAX* was not influenced by

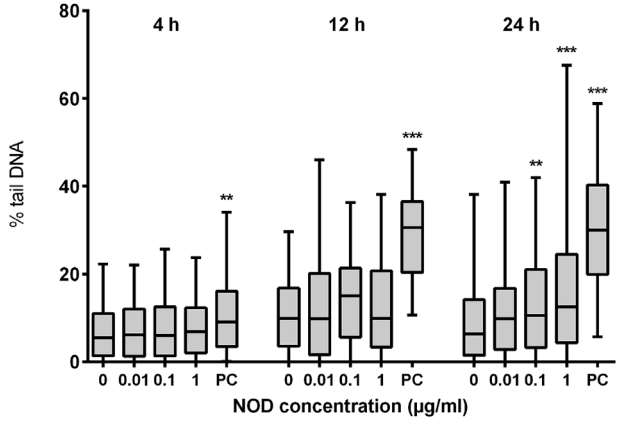

Fig. 4. NOD-induced DNA strand breaks in HepG2 cells. Cells were exposed to NOD (0.01, 0.1 and 1 μg/ml) for 4 h, 12 h and 24 h. Benzo[a]pyrene (30 μM) was used as the positive control (PC). DNA damage was assessed with the comet assay and is expressed as percent of tail DNA. Fifty nuclei were analyzed 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 NOD-treated cells and the vehicle control (0) is indicated by *P < 0.05, **P < 0.01, and ***P < 0.001.

NOD at tested concentrations and exposed times. The relative expression of mRNA of oxidative stress responsive genes such as *GPX1* and *GCLC* were down-regulated after short exposure times (4 and 12 h), while after 24 h of exposure both genes were significantly and dose-dependently up-regulated. The expressions of other selected genes involved in the response to oxidative stress (*CAT*, *SOD1* and *GSR*) were not affected by NOD at applied conditions.

4. Discussion

It is considered that NOD acts predominantly as tumor promoter via inhibition of protein phosphatases 1 and 2A (Yoshizawa et al., 1990); however, the evidence is accumulating that it is genotoxic and is therefore suspected to be a human carcinogen. NOD has been classified by the International Agency for Research on Cancer (IARC) as "animal carcinogen but not classifiable as to its carcinogenicity to humans" (Group 3), mostly due to the lack of sufficient experimental data.

In the present study genotoxic activity of NOD and its induction of oxidative stress were studied in HepG2 cells. At non-cytotoxic concentrations NOD did not increase the level of DNA double strand breaks, while the levels DNA single strand breaks and Fpg-sensitive sites were statistically significantly elevated due to oxidative damage. These results are consistent with previous publications, reporting that NOD induces oxidative stress (Lankoff et al., 2002) and produces increased intracellular ROS, which is associated with modulation of intracellular glutathione content and lipid peroxidation (Bouaïcha and Maatouk, 2004). Previously, NOD induction of oxidative DNA damage in HepG2 cells has already been shown; however at much higher concentrations (1–10 μg/ml) (Lankoff et al., 2006) than used in our study. In addition, NOD (2 and 10 ng/ml) induced time and dose dependent formation of 8-oxo-dG adducts in primary cultured rat hepatocytes (Maatouk et al., 2004). The results of our study showed that oxidative DNA damage induced by NOD was the most pronounced after short time exposure (4 h) being significant already at the lowest tested concentration (0.01 μg/ml). The amount of Fpg-sensitive sites gradually declined with time but remained significantly elevated also after 12 and 24 h at higher NOD concentrations. In contrast to these results Lankoff et al. reported the highest level of oxidized purines after 24 h exposure but at much higher concentrations (2.5, 5 and 10 μg/ml) (Lankoff et al., 2006).

Genotoxic potential of NOD was further evaluated using the cytokinesis block micronucleus (CBMN) cytome assay. This method enables simultaneous detection of multiple parameters associated with chromosomal instability: micronuclei (MNi) induction that reflects structural and numerical chromosomal aberrations, nucleoplasmic bridges (NPB) formations that are an indicator of chromosomal rearrangements, DNA miss-repair, or telomere end-fusions, and nuclear buds (NPBs) that are considered as an indicator of gene amplification and/or DNA repair complexes (Fenech, 2006). Previously it has been reported that NOD induces the formation of micronuclei in HepG2 cells at 5 and 10 μg/ml after 24 h of exposure and at 2.5 and 5 μg/ml after 48 h. The

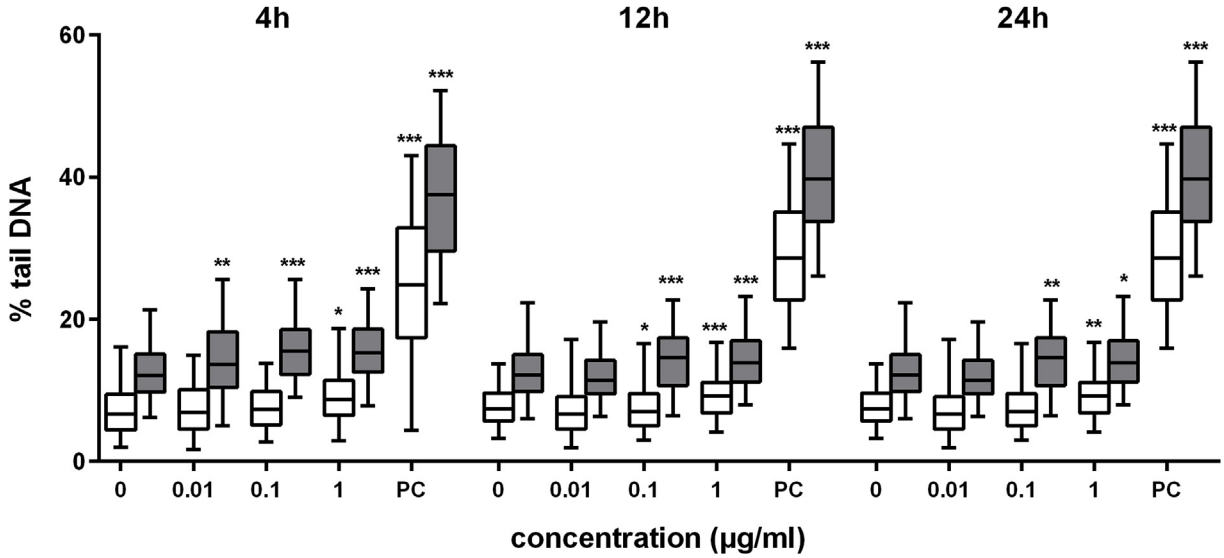

Fig. 5. Induction of oxidized purines in HepG2 cells by NOD. The level of NOD-induced DNA strand breaks without (white box plots) and with formamidopyrimidine glycosylase (Fpg) digestion (gray box plots). The cells were exposed to NOD (0.01, 0.1 and 1 μg/ml) for 4, 12 and 24 h, then the modified comet assay was performed. Tert-butyl hydroperoxide (0.5 mM) was used as the positive control (PC). The levels of DNA strand breaks and oxidized purines are expressed as percent of tail DNA (% tail DNA). Fifty nuclei 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 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.

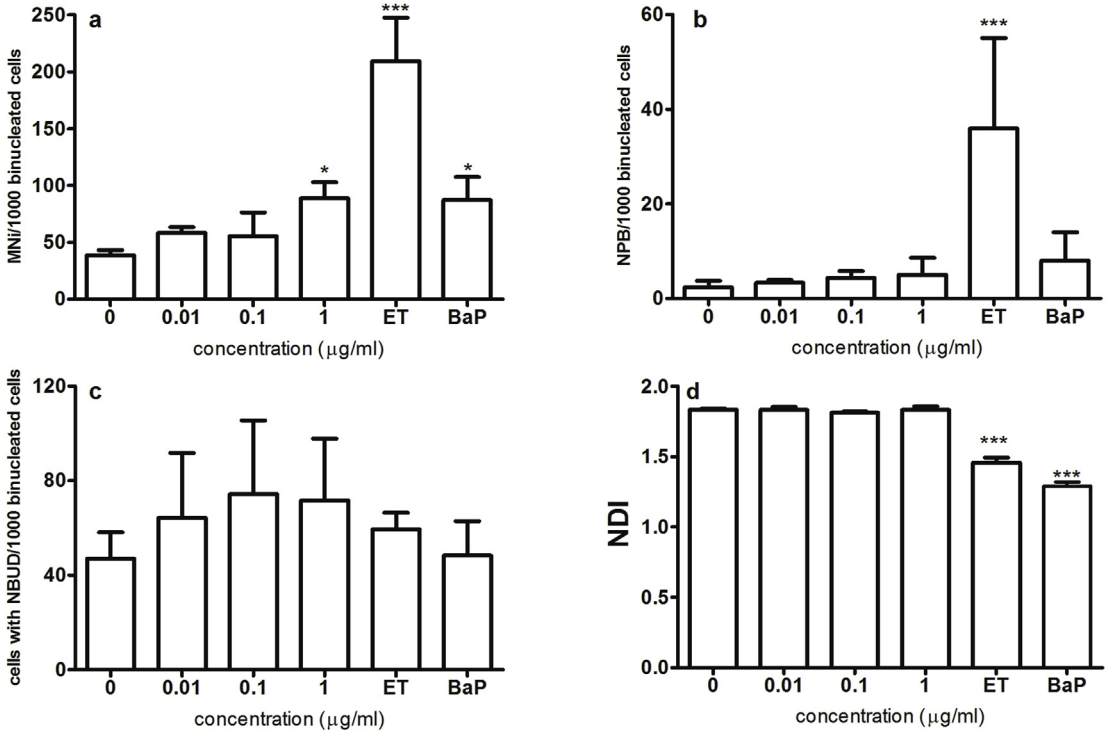

Fig. 6. NOD influence on the frequency of MNi (a), NPBs (b), NBUDs (c) and NDI (d) in HepG2 cells. Cells were exposed to NOD (0.01, 0.1 and 1 μg/ml) for 24 h. Etoposide (ET; 1 μg/ml) and benzo[a]pyrene (BaP; 10 μM) were used as positive controls. Thousand binucleated cells were counted at each experimental point. The NDI was estimated by scoring 500 cells with one to four nuclei. The results of three independent experiments are expressed as mean values ± STD. Significant difference (chi-square test) between NOD-exposed cells and the vehicle control (0) is indicated by *P < 0.05, **P < 0.01, and ***P < 0.001.

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 an aneugen (Lankoff et al., 2006). In the present study we showed that NOD induced the formation of micronuclei already at 1 μg/ml after 24 h. To our knowledge the present study is the first where in addition to MNi the influence of NOD on nuclear buds (NBUDs) and nuclear bridges (NPBs) formation was assessed. After 24 h exposure slight; however, not statistically significant increase in the formation of NBUDs and NPBs was detected pointing out that NOD can potentially lead to the formation of acentric chromosome fragments, dicentric chromosomes or elimination of DNA repair complexes.

In the present study we applied the toxicogenomic approach to evaluate the influence of NOD on the genome level. By toxicogenomic analyses it is possible to identify the genotoxic and carcinogenic hazard and assess the risk of studied substances (Ellinger-Ziegelbauer et al., 2009). The changes in the mRNA expression of *TP53* and its regulated genes such as DNA-damage responsive genes (*CDKN1A*, *MDM2* and *GADD45A*), can be considered as markers of genotoxic and carcinogenic stress (Vogelstein et al., 2000). Moreover, toxicogenomic analyses can be used for the identification of molecular pathways involved in the adverse effects of studied compounds. To date limited data describing the influence of NOD on the mRNA expression has been published. In the liver of exposed F344 rats, strong up-regulation of 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* after the exposure to single i.p. dose NOD (50 mg/kg bw, isolated from pure laboratory cultures of *N. spumigena* L575) was detected (Ohta et al., 1994), which was later confirmed in primary cultured rat hepatocytes treated with 1 μM NOD (Sueoka et al., 1997). To the best of our knowledge our study is the first where changes in the expression of *TP53* and its downstream regulated DNA damage response genes *CDKN1A, GADD45α*, *MDM2*, *BAX* and *BCL2* were measured. The results revealed that NOD induced significant changes in the expression of the selected genes involved in the DNA damage response, which indicated the activation of cell defence against NOD-induced DNA damage in HepG2 cells.

The tumor-suppressor gene, *TP53*, plays central crucial role in the cellular response to agents or conditions that damage DNA by activating the transcription of many essential genes controlling DNA repair, cell cycle arrest, differentiation, senescence and apoptosis (Vogelstein et al., 2000). Although no changes in the expression of *TP53* at the mRNA level were detected due to NOD exposure, this is not unusual, as DNA damage can activate the p53 protein through its phosphorylation by DNA damage-responsive kinases and, to minor extent, through deregulation of gene expression (Zhou and Elledge, 2000). Very recently Faltermann et al. (2016a) reported no alterations in transcript level of *p53* in adult zebrafish liver organ cultures *in vitro*. Similar results were obtained for MCLR where mRNA level of *TP53* was increased only after 2 h of exposure, while from 4 h onwards no induction of *TP53* at the mRNA level was detected in HepG2 cells (Zegura et al., 2008). Therefore, according to our results it is not possible to conclude whether NOD influenced the p53 pathway. MDM2, E3 ubiquitin ligase, mediates ubiquitination of p53 by forming a complex with p53 and thereby inhibiting its cellular functions and targeting it for degradation by ubiquitin-mediated proteolysis (Wu et al., 2001). It is up-regulated by elevated levels of p53 (Vogelstein et al., 2000). The results showed that *MDM2* was significantly up-regulated only after 24 h of exposure to 0.1 μg/ml of NOD.

The main target of P53 due to DNA damage is *CDKN1A* that encodes $P21^{WAF1/CIP1}$, an inhibitor of cyclin-dependent kinases (CDKs). Another gene, *GADD45A*, involved in DNA damage response can be induced by genotoxic stress through P53-dependent and -independent pathways (Smith et al., 1994). *CDKN1A* and *GADD45α* are both associated with the cell-cycle arrest upon DNA damage in G1 and G2-M phase of the cell-cycle, respectively (Waldman et al., 1995; Zhan, 2005). Their proteins play important role in DNA repair process (Hollander et al., 2001; Smith et al., 1994). After 4 h of exposure down-regulation of *CDKN1A* and *GADD45α* was observed at the mRNA level. To our

Table 1
Changes in the 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	NOD concentration (μg/ml)	4 h	12 h	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*****
CAT	0	1.00 ± 0.01	1.00 ± 0.03	1.00 ± 0.05
	0.01	0.88 ± 0.01	0.95 ± 0.03	0.99 ± 0.03
	0.1	0.94 ± 0.01	0.88 ± 0.03	1.10 ± 0.03
	1	1.03 ± 0.02	0.96 ± 0.02	1.03 ± 0.04
	PC	0.89 ± 0.01	**0.67 ± 0.01****	**0.26 ± 0.02****
SOD1	0	1.00 ± 0.01	1.00 ± 0.04	1.00 ± 0.11
	0.01	0.95 ± 0.05	0.98 ± 0.02	1.19 ± 0.02
	0.1	0.91 ± 0.07	1.00 ± 0.02	1.36 ± 0.21
	1	0.92 ± 0.04	0.91 ± 0.08	1.06 ± 0.02
	PC	0.89 ± 0.02	1.06 ± 0.08	1.32 ± 0.08
GPX1	0	1.00 ± 0.01	1.00 ± 0.06	1.00 ± 0.02
	0.01	0.99 ± 0.17	0.86 ± 0.01	**1.21 ± 0.04***
	0.1	**0.83 ± 0.02***	**0.78 ± 0.01***	**1.34 ± 0.08***
	1	0.90 ± 0.02	**0.80 ± 0.01***	**1.27 ± 0.11***
	PC	0.94 ± 0.02	0.98 ± 0.02	**3.03 ± 0.41***
GR	0	1.00 ± 0.02	1.00 ± 0.01	1.03 ± 0.34
	0.01	1.08 ± 0.11	0.94 ± 0.01	1.00 ± 0.13
	0.1	0.85 ± 0.02	0.85 ± 0.04	0.99 ± 0.03
	1	1.02 ± 0.05	0.91 ± 0.01	0.91 ± 0.03
	PC	0.95 ± 0.03	1.08 ± 0.05	0.85 ± 0.08
GCLC	0	1.00 ± 0.04	1.00 ± 0.02	1.00 ± 0.01
	0.01	0.94 ± 0.02	0.91 ± 0.04	1.10 ± 0.07
	0.1	**0.80 ± 0.01***	**0.73 ± 0.06***	**1.17 ± 0.03***
	1	1.03 ± 0.03	**0.83 ± 0.01****	**1.53 ± 0.19***
	PC	1.07 ± 0.01	1.32 ± 0.26	1.12 ± 0.26

The asteriks denote a more than 1.2-fold significant difference 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, where the cells were exposed to BaP 30 μM.

knowledge there are no literature data on their activity or protein levels after NOD exposure, which has to be further elucidated. The pattern of mRNA levels of *CDKN1A* after NOD exposure differs from that observed in MCLR exposed HepG2 cells. Namely MCLR induced dose- and time-dependent increase in the expression of *CDKN1A* gene (Zegura et al., 2008), which is not the case for NOD. On the contrary the results for mRNA level of *GADD45α* follow the similar pattern as those obtained for MCLR (Zegura et al., 2008). After 12 h of NOD exposure significant up-regulation of *GADD45α* was determined already at the lowest studied concentration and was even more pronounced after 24 h exposure indicating the repair of DNA damage induced by NOD. It has been previously reported that NOD interferes with the nucleotide excision repair (NER) in CHO cells (Lankoff et al., 2008) and the authors have suggested that NOD suppresses the incision step through the XPF/ERCC1 complex, which is likely to be one of the mechanisms responsible for NOD induced genome instability. Moreover, considering that oxidative DNA damage is repaired primarily via the BER pathway (Barzilai and Yamamoto, 2004), interference of NOD with BER would provide additional explanation for our results. In the literature it has been reported that structurally similar toxin MCLR, which has extensively been studied, interferes with the base excision repair (BER) in addition to NER (for review see (Zegura et al., 2011)).

Another role of P53 is the regulation of apoptosis by controlling the transcription of anti- and pro-apoptotic genes of BCL2 family (Adams

and Cory, 2007; Hanahan and Weinberg, 2011; Perfettini et al., 2004). The anti-apoptotic BCL2 family members such as BCL2 and BCL-XL maintain cell survival by inhibiting pro-apoptotic BCL2 family members BAX and BAK (Youle and Strasser, 2008). Very recently it was shown that NOD induced apoptosis in primary rat hepatocytes in culture. It significantly elevated caspase activities at ≥100 nM and induced early increase in phosphorylated ERK1/2, p90RSK, p85S6K, p70S6K as well as p38 and a late induction of the anti-apoptotic Bcl-xL (Ufelmann and Schrenk, 2015). In *Carassius auratus* lymphocytes NOD induced apoptosis-related cytotoxic effects via the mitochondrial apoptotic pathway. It down-regulated *bcl-2*, and up-regulated *bax* expression at the mRNA and protein levels; in addition it increased the activities of caspase-3 and caspase-9, but not caspase-8 (Zhang et al., 2012). Recently Rymuszka and Sieroslawska (2018) described that in isolated carp head kidney (HK) leukocytes and common carp (*Cyprinus carpio* L.) leukocyte cell line (CLC) NOD increased caspase 3/7 activity and DNA fragmentation. In the present study, the expression of pro-apoptotic gen *BAX* was not altered by NOD, while after 24 h exposure the expression of anti-apoptotic gene *BCL2* was significantly up-regulated already at ≥0.1 μg/ml. Similarly NOD did not alter the transcript levels of pro-apoptotic genes Bcl-2 like protein 4 (*bax*) in zebrafish eleuthero-embryos and adult zebrafish liver organ cultures (Faltermann et al., 2016b), while in adult zebrafish liver organ cultures it led to a significant transcriptional induction of genes (*bip*, *xbp-1s*, *chop* and *atf4*) that are involved in endoplasmic reticulum (ER)-stress, which is related to apoptosis. Despite the indications that NOD induces apoptosis the role of anti-apoptotic and pro-apoptotic BCL members involved in NOD induced apoptosis needs to be further elucidated, especially on the protein level.

In the present study it has been clearly shown that NOD induced oxidative stress, therefore it is expected to trigger the cellular anti-oxidative defence system. In terms of this we measured the gene expression of the most important antioxidant enzymes, namely glutathione peroxidase (GPX), glutathione reductase (GR), glutamate-cysteine ligase (GCLC), catalase (CAT) and superoxide dismutase (SOD). A crucial role in intracellular antioxidant defence systems plays a glutathione, which can exist in either a reduced (GSH) or oxidized (GSSG) form. GPX1–4 catalyze the reduction of organic hydroperoxides or hydrogen peroxide where upon glutathione disulfide (GSSG) is formed. GSSG can be subsequently reduced back to GSH by GR (Jones, 2002). Our results revealed that NOD down-regulated the expression of *GPX* after 12 h of exposure, while after longer exposure (24 h) it significantly up-regulated the mRNA level of *GPX*. The expression of *GR* was not affected by NOD at measured time points. Furthermore, we measured the mRNA level of glutamate-cysteine ligase (GCLC), which is one of the two crucial ATP-dependent enzymes that are responsible for *de novo* synthesis of GSH. In HepG2 cells *GCLC* was down-regulated after short time exposure to NOD and significantly up-regulated after 24 h of exposure to NOD (1 μg/ml), showing potential *de novo* synthesis of GSH. It is well known that MCs are detoxificated through conjugation to the tripeptide glutathione (GSH) via glutathione-S-transferases (GSTs) (Pflugmacher et al., 1998), while not much is known on NOD detoxification pathways. To date, only a few studies have reported nodularin-GSH and nodularin-Cys conjugates in tissue from mussels (Sipia et al., 2002), brine shrimp *Artemia salina* (Beattie et al., 2003) and freshwater shrimp *Palaemonetes argentinus* (Galanti et al., 2013). Vuorinen et al., (2009) reported that in adult flounder exposed to *Nodularia* slurry no GSH conjugates were observed in the liver, suggesting a rapid excretion/disintegration of NOD detoxification products, while in bile of exposed fish the presence of NOD-like compounds was reported. Increased levels of GST were reported for *Gammarus oceanicus* (Turja et al., 2014) and brine shrimp *Artemia salina* (Beattie et al., 2003) exposed to NOD rich extracts and purified NOD, respectively, suggesting potential detoxification pathway of NOD. In addition, also CAT, SOD and GPx activities were elevated in *Gammarus oceanicus* (Turja et al., 2014), while in opposite to this NOD and *Nodularia spumigena* extract decreased CAT activities in european flounder (*Platichthys flesus*) (Persson et al., 2009) and blue mussels (*Mytilus edulis*) (Kankaanpää et al., 2007), respectively.

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 as the toxin induced increased ROS production and oxidative damage as well as deregulated oxidative stress induced genes in HepG2 cells. Moreover, NOD exerted effects comparable to those induced by MCLR; it increased ROS formation, oxidative DNA damage, DNA strand breaks, although some differences have been observed in the kinetics of oxidative DNA damage formation and gene expressions between MCLR and NOD. In addition to DNA strand breaks NOD induced increased formation of micronuclei suggesting its clastogenic/aneugenic activity, which can lead to the accumulation of genetic alterations and consequently may be implicated in carcinogenesis. These results present valuable information on the potential hazard of NOD, especially as the adverse effects were detected at concentrations that have been detected in environmental samples (Barda et al., 2015), including aquatic organisms used as a sources of food or feed.

Declaration of interest

The authors report no conflict of interest.

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