Nodularin Induces Tumor Necrosis Factor-alpha and Mitogen-Activated Protein-Kinases and Leads to Induction of Endoplasmic Reticulum Stress

Nicole Meili^{*,‡}, Verena Christen^{*,‡}, and Karl Fent ^{*,†}

^{*} University of Applied Sciences and Arts Northwestern Switzerland, Gründenstrasse 40, CH-4132 Muttenz, Switzerland, and

[†] Swiss Federal Institute of Technology Zürich, Department of Environmental Systems Science, CH-8092 Zürich

^{*}Corresponding author: Karl Fent, phone: +41 61 467 4571; fax: +41 61 467 47 84; e-mail: karl.fent@fhnw.ch; karl.fent@bluewin.ch.

[‡] Both contributed equally and share first authorship

Running title: Molecular effects of nodularin

ABSTRACT

Nodularin is produced by cyanobacteria *Nodularia spumigena* and of concern due to hepatotoxicity in humans and animals. Here we investigated unexplored molecular mechanisms by transcription analysis in human liver cells, focusing on induction of pro-inflammatory cytokine, the tumor necrosis factor α (TNF- α), endoplasmatic reticulum (ER) stress and components of the activator protein-1 complex in human hepatoma cells (Huh7) exposed to non-cytotoxic (0.1 and 1 μ M) and toxic concentrations (5 μ M) for 24, 48, and 72 h. Transcripts of TNF- α and ER stress marker genes were strongly induced at 1 and 5 μ M at all time-points. TNF- α led to induction of mitogen-activated protein-kinases (MAPK), as demonstrated by induction of *CJUN* and *CFOS*, which form the AP-1 complex. Human primary liver cells reacted more sensitive than Huh7 cells. They showed higher cytotoxicity and induction of TNF- α and ER stress at 2.5 nM, while HepG2 cells were insensitive up to 10 μ M due to low expression of organic anion transporting polypeptides. Furthermore, nodularin led to induction of TNF- α protein, and CCAAT/enhancer-binding protein-homologous (CHOP) protein. Our data indicate that nodularin induces inflammation and ER stress and leads to activation of MAPK in liver cells. All of these activated pathways, which were analyzed here for the first time in detail, may contribute to the hepatotoxic, and tumorigenic action of nodularin.

Key words: nodularin, ER stress response, TNF- α , MAPK signaling

INTRODUCTION

Cyanobacterial blooms occur in eutrophic fresh and brackish waters during warm seasons. About 40 genera, including *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Lyngba*, *Microcystis*, *Nostoc* and *Oscillatoria* can produce toxins (Dawson, 1998). Among them are members of the cyclic peptide families of microcystins (MCs) and nodularins. Both are hepatotoxins that have the amino acids (all-S, all-E)-3-Amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda) in common, which is unique for cyanobacteria. Nodularin consists of five amino acids and about seven variants are known (Pearson et al., 2010). It is produced by *Nodularia spumigena* that lives in brackish waters. In the Baltic Sea up to 18 mg nodularin/g (dry weight) of *Nodularia spumigena* has been found in phytoplankton samples (Kononen et al., 1993), and up to 557 µg nodularin/kg in the liver of European flounders (Persson et al., 2009). In addition, nodularin may enter the food-chain (Karjalainen et al., 2005).

Humans may be exposed to nodularin by contaminated drinking water, fish or seafood (mussels). Nodularin enters cells by active transport via transmembrane multispecific organic anion transporters (OATPs), expressed in liver, kidneys, gastrointestinal tract and brain (Van Apeldoorn et al., 2007). Nodularin is a strong hepatotoxin (Carmichael, 1997; Sivonen, K., Jones, 1999), has tumor promoting activity (Zegura et al., 2008), and is suspected to induce hepatocellular carcinoma (Ohta et al., 1994).

An important molecular toxicological mechanism of these toxins is inhibition of serine/threonine specific protein phosphatases (PP) PP1 and PP2A (Dawson, 1998; MacKintosh et al., 1990; Yoshizawa et al., 1990). Inhibition promotes hyperphosphorylation of proteins, resulting in deterioration of cellular integrity, apoptosis, liver necrosis and hepatic haemorrhage (Batista et al., 2003; Dittmann and Wiegand, 2006). In contrast to MC-LR, nodularin does not bind covalently to PP1 or PP2A (Lindvall et al., 1997).

In addition, inhibition of PP2A also leads to augmentation of tumor necrosis factor α (TNF- α) production (Cornell et al., 2009), as shown in primary rat hepatocytes (Sueoka et al., 1997). TNF- α is involved in the inflammatory response, apoptosis, cell proliferation and cell differentiation. Induction of TNF- α has downstream effects on mitogen activated-protein kinases

(MAPK) (Goetze et al., 1999). MAPK signalling pathways, which include many proteins like the extracellular signal-regulated kinase1/2 (ERK1/2), p38, cMYC and c-Jun N-terminal kinase (JNK), regulate the activities of many transcription factors. When modified, MAPK signalling leads to altered transcription of genes important for cell cycle regulation. Thus, the MAPK pathway is important in cellular stress and inflammatory response (DiDonato et al., 2012), as well as apoptosis. It plays a role in cancer development by regulation of proto-oncogenes like AP-1 (Karin, 1995; Olivares et al., 2014). AP-1 is an activator protein-1 complex involved in the early development of human hepatocellular carcinoma (HCC) (Liu et al., 2002).

In our previous study, we showed that MC-LR induced ER-stress in human hepatoma cells Huh7 and led to activation of NF κ B pathway (Christen et al., 2013). The ER stress response is activated when folding and export of proteins are perturbed. Accumulation of non- or misfolded proteins in the ER induces the unfolded protein response (UPR) that reduces protein synthesis, enhances production of chaperones that facilitate protein folding and increases proteosomal degradation of misfolded proteins (Harding et al., 2002; Zhang and Kaufman, 2004). There are three distinct pathways triggered by ER stress, namely protein kinase RNA-like ER kinase (PERK), inositol-requiring protein 1 (IRE1) and activating transcription factor 6 (ATF6) pathway; all three pathways are regulated by transmembrane proteins (Rutkowski and Kaufman, 2004). The chaperone immune globulin binding protein (BiP) dissociates from transmembrane proteins PERK, IRE1 and ATF6 and activates the three pathways. PERK phosphorylates eukaryotic elongation factor 2 α (eIF2 α), leading to a general translation block, but to a specific translation of ATF4 (Harding et al., 1999). IRE1 turns X-box binding protein 1 (XBP-1) mRNA into transcription factor XBP-1s. ATF6 gets phosphorylated and turns into an active transcription factor. XBP-1s and ATF6 lead to up-regulation of a wide variety of ER stress target genes, including chaperones like BiP. All three pathways result in up-regulation of CCAAT/enhancer-binding protein-homologous protein (CHOP), which is a pro-apoptotic marker gene. Overexpression of CHOP promotes cell death. ER stress response and associated UPR have important consequences, including apoptosis and play an important role in acute and chronic liver diseases.

Despite the importance of nodularin as a hepatotoxin, the modes of action are poorly known. The aim of our present study was to investigate molecular effects of pathways of nodularin in primary human liver cells and human hepatoma cells. We found that the induction of MAPK and ER stress response are important pathways in the molecular action of nodularin. Additionally, pro-inflammatory cytokines TNF- α and IL8 contribute to the effects.

Materials and Methods

Chemicals

Nodularin (CAS: 118399-22-7) was purchased from Enzo Life Sciences (ELS) AG (Lausen, Switzerland). The antibodies CHOP and Actin were purchased from Thermo Scientific (Reinach, Switzerland), anti-BiP was purchased from Sigma Aldrich (Buchs, Switzerland), respectively. The secondary anti-mouse antibody IRDye 680RD and anti-rabbit antibody IRDye 800CK were purchased from Lucerna-Chem (Lucerne, Switzerland). TNF- α inhibitor sauchinone was purchased from Sigma Aldrich (Buchs, Switzerland).

Cells

The human hepatoma cell line Huh7 and HepG2 were kindly provided by Markus Heim, University Hospital Basel, Switzerland, and Rene Prétôt, FHNW, Switzerland, respectively. Cells were grown in DMEM with GlutaMAX™ (LuBioScience, Lucerne, Switzerland) supplemented with 10% FCS in a humidified incubator with 5% CO₂ at 37 °C. Cells were usually split every 4 days and sub-cultured at split ratios of about 1:6.

ATF-6 transfected Huh7 cells used to perform ATF-6 activity assay were kindly provided by Rene Prétôt, FHNW, Switzerland. The ATF-6 plasmid contains a firefly luciferase gene under the control of the 5x ATF-6 binding sites (response elements) in front of a cfos-promotor. Cells were grown in DMEM with GlutaMAX™ (LuBioScience, Lucerne, Switzerland) supplemented

with 10% FCS in a humidified incubator (5% CO₂, 37°C). Cells were sub-cultured every 3 days at split ratios of 1: 6.

Cryo-conserved human primary liver cells were obtained from hepacult GmbH (Regensburg, Germany) on dry ice. The hepatocytes were isolated on March 17, 2008 from healthy liver tissue of a female, 64 years old donor and kept frozen in liquid nitrogen at -196°C. Cells were thawed and grown in DMEM with GlutaMAX™ (LuBioScience, Lucerne, Switzerland) supplemented with 10% FCS, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, 800 µg/L hydrocortisone, 125 U/L insulin and 7.3 µg/L glucagon in a humidified incubator (5% CO₂, 37°C). For all experiments, human primary liver cells from the same donor and from the same batch were used.

Cytotoxicity

Detailed information about cytotoxicity assay with Huh7 and HepG2 cells can be found in the Supporting Information (SI). Primary liver cells were plated at a density of 300'000 cells per well in 24-well plates. After 4 h, cells were treated with different concentrations of nodularin (0.1-1000 nM) for 24 and 48 h. The [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2 H-tetrazolium (WST-8) cytotoxicity assay was performed as described before (Nakagawa et al., 2002). The absorbance was measured by an automatic microplate reader (GENios Tecan reader, Tecan, Männedorf, Switzerland) at 570 nm. The results were expressed as percent living cells compared to un-treated control cells.

ATF-6 Activity Assay

The stable transfected Huh7 cells were treated with nodularin (0.1-5 µM). After different incubation times (24, 48 and 72 h), luciferase activity was measured using Steady Glo Luciferase assay system (Promega Biosciences, Inc., Wallisellen, Switzerland) according to the manufacturer's instructions.

Gene Expression Analysis: RNA Isolation, Reverse Transcription, and Quantitative (q)PCR

Total RNA was isolated from Huh7 cells, HepG2 cells and primary liver cells using Trizol reagent according to the manufacturer's instructions. RNA was reverse transcribed by Moloney murine leukemia virus reverse transcriptase (Promega Biosciences, Inc., Wallisellen, Switzerland) in the presence of random hexamers (Roche) and deoxynucleoside triphosphate. The reaction mixture was incubated for 5 min at 70 °C and then for 1 h at 37 °C. The reaction was stopped by heating at 95 °C for 5 min. qPCR was performed based on SYBR green fluorescence (SYBR green PCR master mix; Roche). The sequences of the used primers are shown in the supplementary Table S1. The amplification conditions were 95 °C for 5 min for initial denaturing, 40 cycles of 95 °C for 30 s for denaturing, 61 °C for 60 s for annealing and elongation. Melting-curve analyses were performed after each run to ensure the formation of specific products. The difference in the cycle threshold (ΔCT) value was derived by subtracting the CT value for GAPDH, which served as an internal control, from the CT value for the target genes. All reactions were run in duplicates using a BioRad real time PCR machine (CFX 96 Real Time System). mRNA expression levels of target genes were expressed as a several fold increase according to the formula $2^{\Delta CT \text{ (not exposed)} - \Delta CT \text{ (exposed)}}$ in which "not exposed" are solvent control treated samples and "exposed" are nodularin treated samples. Due to the limited amount of RNA of human primary liver cells, not all genes could be analysed in these cells. Quantification of OATP transcripts were made using the relative quantification method and normalized to the housekeeping gene GAPDH with Qgene method (<http://www.qgene.org/qgene/index.php>) (Simon, 2003) according to the equation:

$$MNE = ((E_{ref})^{Ct_{ref}; \text{ mean}}) / ((E_{target})^{Ct_{target}; \text{ mean}})$$

where, MNE stands for mean normalized expression; E_{ref} is housekeeping gene efficiency; E_{target} is target gene efficiency; $Ct_{ref}; \text{ mean}$ is mean Ct value for housekeeping gene; and $Ct_{target}; \text{ mean}$ stands for mean Ct value for target gene. Data are presented as the gene of interest expression relative to the housekeeping gene expression multiplied by the factor 10^4 for better representation. The efficiency of each primer pair was assessed by making a dilution series of male liver

cDNA, which was used to perform qPCR as described above. The efficiency was calculated using Qgene. The efficiency were: *GAPDH*: 2.04; *OATP1A2*: 2.03; *OATP1B1*: 1.94; *OATP1B3*: 1.87; *OATP2B1*: 1.95 and *OCT-1*: 2.

TNF- α Inhibition

Huh7 cells were plated at a density of 1000 000 cells per well in 6-well plates. After 24 h, cells were exposed to 5 μ M nodularin alone, to 30 mM of the TNF- α inhibitor sauchinone alone, or to 5 μ M nodularin and 30 mM sauchinone together for additional 24 and 48 h. Afterwards RNA was isolated and gene expression was analysed as described above.

TNF- α ELISA

The amount of TNF- α in cell culture supernatant of Huh7 cells was measured with Cymax TNF- α human ELISA Kit (Adipogen AG, Liestal, Switzerland) according to the manufacturer's protocol.

Preparation of Cell Extracts and Immunoblotting

Cells were homogenized in 50 μ l of lysis buffer (50 mM Tris, 150 mM NaCl, 15 mM EDTA, 0.1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride) incubated for 20 min on ice, centrifuged at 14,000 rpm for 5 min and the supernatant containing the proteins was transferred into a new 1.5 ml reaction tube. Protein concentrations were determined with Thermo Scientific BCATM protein assay kit (Fish Scientific, Wohlen, Switzerland). Immunoblotting was performed as described. (Duong et al., 2004) To detect the Actin, BiP and the Chop band, membranes were incubated with secondary antibodies IRDye® 650 Goat anti-Rabbit IgG or IRDye® 650 Goat anti-Mouse IgG from LICOR Biotechnology - GmbH, Bad Homburg, Germany and scanned with Odyssey CLx (LICOR Biotechnology - GmbH, Bad Homburg, Germany).

Statistical analysis

For curve fitting, EC_{50} calculation, the percentage of living cells versus the logarithm of concentrations was plotted. To fit dose response curves the four-parameter logistic equation (Hill equation) was applied. Differences between treatments were assessed by one way ANOVA followed by a Bonferroni's multiple comparison test to compare treatment means with respective controls. Results are given as means $\pm$ standard error of means. Differences were considered statistically significant at $0.05 > p > 0.01$ (represented by one asterisk), $0.01 > p > 0.001$ (two asterisks), and at $0.001 > p > 0.0001$ (three asterisks).

RESULTS

Cytotoxicity, Expression of OATPs and Induction of Apoptosis

We compared the cytotoxicity of nodularin between two human hepatoma cell lines, Huh7 and HepG2, and human primary liver cells after 24 h exposure (Figure 1A). Cytotoxicity occurred in Huh7 cells (EC_{50} value of 11.28 μ M), whereas no significant toxicity occurred in HepG2 cells. Human primary liver cells reacted much more sensitive (EC_{50} value of 4.41 nM). Exposure of for 48 and 72 h induced no cytotoxicity in HepG2 cells, whereas cytotoxicity occurred in Huh7 cells (EC_{50} value of 4.09 μ M after 48 h, and 3.09 μ M after 72 h) (Fig. S1).

Organic anion transporting polypeptides OATP1B1, OATP1B3, and OATP1A2 transporters are responsible for active uptake of MCs into human cells (Fischer et al., 2005), and similarly, oatp1d1 for nodularin in zebrafish cells (Faltermann et al., 2016). Thus, we assessed the basal expression of *OATP1A2*, *OATP2B1*, *OATP1B2*, *OATP1B3* and *OCT-1* in these cells. Figure 1B shows that Huh7 cells and human primary liver cells have similar expression levels for *OATP1B1* and *OATP1B3*, while HepG2 cells showed only very weak expression. *OATP2B1*, which is not involved in uptake of cyanobacterial toxins, was expressed equally in all three cell lines. *OATP1A2* showed only very weak expression in HepG2 compared to primary human liver cells and Huh7 cells. *OCT-1* was highly expressed in primary liver cells (Figure 1B). This suggests that cytotoxicity in the different cells was related to the occurrence of uptake transporters OATP1B1 and OATP1B3, while OATP1A2 played a minor role.

To analyse for apoptosis, two apoptotic marker genes, *DR5* and *CASPASE 8*, were analysed in Huh7 cells after exposure to 0.1, 1 and 5 μ M nodularin. There was a strong and significant dose-dependent induction of *DR5* in Huh7 cells and a significant but much weaker induction of *CASPASE 8* (Figure 1C). *DR5* was also significantly induced in human primary liver cells after exposure to 2.5 nM nodularin for 24 h (Figure 1D).

Induction of Pro-Inflammatory Cytokines *TNF- α* and *IL8*

As cyanobacterial toxins induce pro-inflammatory cytokines, Huh7 cells and human primary liver cells were exposed to different concentrations of nodularin (Huh7 cells: 0.1, 1 and 5 μ M for 24, 48 and 72 h; and human primary liver cells: 1 and 2.5 nM for 24 h) to analyse the expression of pro-inflammatory cytokines *TNF- α* and *IL-8*. We found a strong, significant and dose-dependent induction of *TNF- α* and *IL-8* in Huh7 cells (Figure 2A). Similarly, in primary liver cells *TNF- α* and *IL8* were induced following exposure to 2.5 nM nodularin for 24 h (Figure 2B). In addition, we measured *TNF- α* in the supernatant of nodularin exposed Huh7 cells (Fig. 2C). We found a dose- and time-dependent up-regulation of *TNF- α* protein. Highest *TNF- α* induction was detected in the supernatant at the concentration of 5 μ M nodularin and 48 h exposure. The protein data of *TNF- α* clearly reflects the *TNF- α* mRNA data (Fig. 2A and C).

Activation of MAPK Signalling

TNF- α can activate the MAPK signalling pathway. Therefore, we analysed the induction of ERK1/2 regulated kinases *CMYC* and *FRA1*, p38 regulated kinase *CFOS* and the JNK regulated kinases *CJUN* and *JUND* in Huh7 and human primary liver cells after exposure to nodularin (Huh7 cells: 0.1, 1 and 5 μ M nodularin for 24, 48 and 72 h; and human primary liver cells: 1 and 2.5 nM nodularin for 24 h). We found a very strong and dose-dependent induction of *CFOS* and *FRA1* in Huh7 cells upon nodularin exposure (Figure 3A). Additionally, *CMYC*, *CJUN* and *JUND* showed a dose-dependent and significant induction (Figure 3A). Induction of MAPK pathway was also analyzed in human primary liver cells by assessing transcriptional alterations

of *CFOS*, *FRA1*, and *JUND*. We found an up-regulation of *FRA1* and *JUND* already at 1 nM, while induction of *CFOS* occurred at 2.5 nM (Figure 3B).

Induction of ER Stress Response

As MC-LR and TNF- α can lead to induction of ER stress, we analysed the expression of ER stress marker genes *BIP*, *XBP1s*, *ATF4*, *CHOP* and *GADD34* in Huh7 cells and human primary liver cells upon exposure to nodularin. All of these ER stress markers showed a significant dose-dependent induction in Huh7 cells (Figure 4A). The strongest induction showed *GADD34*, while weakest but significant induction occurred for *BIP* (Figure 4A). In human primary liver cells transcripts of *XBP1s* and *GADD34* showed a concentration-dependent up-regulation, while transcripts of *BIP* and *CHOP* did not (Fig. 4B).

In addition to transcripts, we analysed BIP and CHOP expression on the protein level. As shown in Figure 4C, there was a strong induction of CHOP in Huh7 cells after 24 h exposure to 5 μ M nodularin.

To investigate the induction of the ATF-6 pathway upon nodularin exposure, the activation of transcription factor ATF-6 was analysed in Huh7 cells transfected with a plasmid containing a *cfos* promoter, which was under the control of an ATF-6 response element, and which regulated the expression of luciferase. We found a dose-dependent activation of ATF-6 following a 24 h exposure to nodularin (Figure S2). After 48 h, ATF-6 was significantly activated upon exposure to 5 μ M nodularin (Figure S2). Along with transcription analysis, these data indicate that nodularin induced ER stress and associated UPR.

Co-Exposure of Huh7 Cells to Nodularin and TNF- α Inhibitor Sauchinone

To identify and investigate TNF- α -dependent effects of nodularin, Huh7 cells were co-exposed to nodularin and to the TNF- α inhibitor sauchinone (Lee et al., 2003). Co-exposure of Huh7 cells to nodularin and sauchinone prevented the induction on of *TNF- α* and *IL8*, the latter regulated by TNF- α on mRNA level (Figure 5A). In addition, TNF- α protein in the supernatant was measured after exposure to nodularin alone, and after co-exposure to nodularin and sauchinone. Af-

ter 24 h co-exposure, the amount of TNF- α protein is already reduced, and after 48 h co-exposure, even no TNF- α was detected in the cell supernatant (Figure 5B). These TNF- α protein data clearly reflect the obtained TNF- α mRNA data (Figure 5A and B). The nodularin-related induction of the MAPK *CFOS* and *JUND* was completely prevented by sauchinone (Figure S3A). The induction of *FRA1* was reduced, whereas *CMYC* and *CJUN* transcripts were not altered (Figure S3A). In contrast, inhibition of TNF- α by sauchinone had no effect on the induction of ER stress by nodularin (Figure S3B). However, inhibition of TNF- α also reduced induction of the apoptotic genes *DR5* and *CASPASE 8* (Figure S3C).

DISCUSSION

Cytotoxicity, OATPS and Apoptosis

In Huh7 and human primary liver cells, nodularin induced dose-dependent cytotoxicity. The EC₅₀ (24 h) values for Huh7 cells and primary liver cells were 11.28 μ M and 4.41 nM, respectively, which demonstrates the much higher sensitivity of primary liver cells. In contrast, nodularin caused almost no cytotoxicity in HepG2 cells (Figure 1). Cellular uptake of MC-LR occurs via active transport by OATP1A2, OATP1B1 and OATP1B3 (Fischer et al., 2005). MC-LR and nodularin have a similar size and structure, thus uptake of nodularin is suggested to occur similarly by these transporters, which is further indicated by the role of *oatp1d* in nodularin uptake in zebrafish (Faltermann et al. 2016). We therefore investigated expression of *OATP1A2*, *OATP2B1*, *OATP1B1*, *OATP1B3* and *OCT-1*. HepG2 cells showed low expression of *OATP1A2*, *OATP1B1* and *OATP1B3*. These transporters are suggested to be responsible for the up-take of nodularin and consequently, lack of cytotoxicity in HepG2 cells could be related to low occurrence of these OATPs. In contrast, in human primary liver cells *OATP2B1*, *OATP1B3* and *OCT-1* transcripts showed highest abundance. The much higher sensitivity of primary cells compared to Huh7 cells could be related to the higher occurrence of these transporters.

Nodularin led to a significant up-regulation of *DR5* at all concentrations. *DR5* is a target of CHOP (Chen et al., 2007), and its over-expression sensitizes cells to apoptotic death (Kim et al., 2006). The correlation between *DR5* and CASPASE 8 is given by the fact, that *DR5* siRNA knock-down strongly inhibited caspase activation and apoptosis in response to various ER stressors (Lu et al., 2014). Therefore, our data not only indicate that cytotoxicity of nodularin is dependent on the occurrence of specific OATPs, but also for cellular activity, including up-regulation of apoptosis, and induction of ER stress, TNF- α and activation of MAPK.

Inflammation

The strong induction of *TNF- α* and *IL8* suggests that cytokines play an important role in the mode of action of nodularin (Figure 2A). In human primary liver cells induction occurred already at 2.5 nM. TNF- α belongs to a group of cytokines, which are involved in the pathogenesis of several diseases including cancer (Locksley et al., 2001). TNF- α can also act as an endogenous tumor promoter (Fujiki and Suganuma, 1994). Additionally, TNF- α regulates the expression of various other cytokines, such as interleukin 8 (IL8) (Osawa et al., 2002). Furthermore, cytokines trigger ER stress (Denis et al., 2010; Hotamisligil, 2010). Our data with the TNF- α inhibitor sauchinone further substantiated the important role of TNF- α in the action of nodularin (Figure 5). Decreased expression of TNF- α also affected downstream events, including MAPK and *DR5* (apoptosis). Our data indicate that induction of TNF- α represents the initial molecular effect of nodularin, which subsequently leads to activation of MAPK and ER stress.

MAPK Pathway

Our data demonstrated that *CFOS*, *FRA1*, *CMYC*, *CJUN* and *JUND* were significantly up-regulated in Huh7 cell line upon nodularin exposure (Figure 3A). In primary liver cells significant induction of *CFOS*, *FRA1* and *JUND* occurred at 1 and 2.5 nM nodularin, respectively (Figure 3B). TNF- α engages multiple signal transduction pathways, including three groups of MAPK, the extracellular-signal-regulated kinases (ERKs), cjun NH2-terminal kinases (JNKs), and p38 MAP kinases. MAPK itself induces the expression of TNF- α and contributes to the biological activity

(Sabio and Davis, 2014). Our experiments with the TNF- α inhibitor sauchinone showed a significant down-regulation of *CFOS*, *FRA1* and *JUND* (Figure S3A) in response to nodularin. This is consistent with data in murine embryo fibroblasts, where TNF- α was a key mediator in triggering the JNK pathway (Ventura et al., 2003). Therefore, the MAPK is activated via induction of TNF- α by nodularin.

Activator protein-1 (AP-1) is a transcription factor building a complex with one member of the FOS family (*CFOS*, *FRA1*), and one member of the JUN family (*CJUN*, *JUND*) upon activation (Bohmann et al., 1987; Ryder et al., 1989). AP-1 is involved in the primary cellular response to growth factor stimuli (Angel and Karin, 1991). A close correlation was found between *JUND* and *CFOS* levels and AP-1 binding activities in a tumoral stage of liver cells. Thus, the AP-1 complex seems an early event in development of human hepatocellular carcinoma (Liu et al., 2002). Our data therefore suggest early events in the tumorigenic action of nodularin.

ER Stress

In our previous study, we found that MC-LR led to induction of ER stress and associated UPR. Persistent ER stress leads to downstream processes like inflammation and induction of the MAPK (Christen et al., 2014). Similarly, our present study demonstrates that nodularin induced ER stress in Huh7 and primary liver cells. We investigated all three ER stress response pathways in detail and found an induction of *BiP* on the transcription and protein level, as well as induction of the transcription factor *ATF4*. Additionally, activation of *GADD34* occurred (Figure 4A). *GADD34* is a protein phosphatase that enhances the de-phosphorylation of the initiation factor eIF2 α (Novoa et al., 2001), which is involved in the UPR. Furthermore, the IRE1 pathway, resulting in the splicing of *XBP1*, was activated at 1 and 5 μ M nodularin (Figure 4A).

Persistent ER stress has significant consequences and results in the activation of apoptotic pathways. The pro-apoptotic marker gene, *CHOP*, is inducible by all three ER stress sensors (Malhi and Kaufman, 2011). *CHOP* was elevated in Huh7 cells on the mRNA and protein level (Figures 4A, 4C). Our results in primary liver cells also showed induction of *XBP1S* and *GADD34* (Figure 4B). Additionally, ATF-6 was induced in the transactivation assay (Figure S2).

These data indicate that nodularin led to induction of ER stress in Huh7 and primary liver cells. This is similar to the action of MC-LR, although the potency of nodularin was weaker.

Comparison of Nodularin and MC-LR and Reaction between Liver Cells

The molecular effects of nodularin were characterized by induction of the pro-inflammatory cytokines TNF- α and IL8, which in turn triggered the activation of the MAPK pathway. Subsequently, nodularin led to induction of ER stress with associated UPR. Furthermore, nodularin inhibits protein phosphatases. All of these molecular effects contribute to the hepatotoxic effect of nodularin (Figure 6).

The molecular effects of MC-LR and nodularin are similar, although the potency and specific action varies. MC-LR activates the inflammation pathway via NF κ B, INF- α and several IFN- α -stimulated genes, whereas nodularin acts via TNF- α , which in turn induces the expression of IL8 and MAPK. Consequently, both cyanotoxins display hepatotoxic, inflammatory, and tumorigenic actions.

Our data also demonstrated that Huh7 cells exhibited the same molecular effects as human primary liver cells, and therefore act as a good model for liver cells. Due to ethical reasons, studies in cell lines such as Huh7 are favored.

Notes

The authors declare no competing financial interests.

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Figure legends:

Figure 1

Cytotoxicity of nodularin (A) and expression of OATPs (B) in Huh7, HepG2 and human primary liver cells; mRNA levels of DR5 and CASPASE 8 in nodularin-exposed Huh7 cells (C) and mRNA level of DR5 in nodularin-exposed human primary liver cells (D). A: Huh7 (blue), HepG2 (green) and primary human liver cells (purple) were exposed to different concentrations of nodularin for 24 h followed by MTT assay for Huh7 and HepG2 and WST8 assay for primary human liver cells. Shown are results of three independent experiments for Huh7 and HepG2 cells and of two independent experiments for human primary liver cells. B: Basic expression of 5 different OATPs in Huh7 (white), HepG2 (black) and human primary liver cells (grey). The expression levels of different OATPs in HepG2 and human primary liver cells were normalized to expression levels in Huh7 cells. Shown are results of three independent experiments for Huh7 and HepG2 cells and of two independent experiments for human primary liver cells. C: Huh7 cells exposed to 0.1 (light blue bars), 1 (blue bars) and 5 μ M nodularin (dark blue bars) for 24, 48 and 72 h followed by detection of mRNA levels of *DR5* and *CASPASE 8*. Due to toxicity, the 5 μ M nodularin exposure was only done for 24 and 48h. Shown are results of three independent experiments for Huh7. D: Primary liver cells were exposed to 1 (green bars) and 2.5 nM nodularin (blue bars) for 24 h followed by determination of *DR5* mRNA levels. Shown are results of two (1 nM) or three (2.5 nM) independent experiments. Significant differences with p-value of ≤ 0.05 are marked with asterisks.

Figure 2

Induction of inflammatory response in Huh7 and human primary liver cells upon nodularin exposure. A: Huh7 cells exposed to 0.1 (light blue bars), 1 (blue bars) and 5 μ M nodularin (dark blue bars) for 24, 48 and 72 h followed by determination of mRNA levels of *TNF- α* and *IL8*. Due to toxicity, 5 μ M nodularin exposure was not done at 72 h. Shown are results of three independent experiments. B: Primary liver cells were exposed to 1 (green bars) and 2.5 nM nodularin (blue bars) for 24 h followed by determination of mRNA levels of *TNF- α* and *IL8*. Shown are results of

two (1 nM) or three (2.5 nM) independent experiments. C: TNF- α protein level in the supernatant of Huh7 cells exposed to 1 μ M (light blue) and 5 μ M (dark blue) nodularin for 24 h and 48 h. Shown are results of three independent experiments. Significant differences with p-value of ≤ 0.05 are marked with asterisks.

Figure 3

Activation of MAPK signalling in Huh7 and human primary liver cells upon nodularin exposure. A: Huh7 cells were exposed to 0.1 (light blue bars), 1 (blue bars) and 5 μ M nodularin (dark blue bars) for 24, 48 and 72 h followed by determination of mRNA levels of *CFOS*, *FRA1*, *CMYC*, *CJUN* and *JUND*. Due to toxicity, the 5 μ M nodularin exposure was only done for 24 and 48 h. Shown are results of three independent experiments. B: Primary liver cells were exposed to 1 (green bars) and 2.5 nM nodularin (blue bars) for 24 h followed by determination of mRNA levels of *CFOS*, *FRA1*, *CMYC*, *CJUN* and *JUND*. Shown are the results of two (1 nM) or three (2.5 nM) independent experiments. Significant differences with p-value of ≤ 0.05 marked with asterisks.

Figure 4

Induction of ER stress response in Huh7 and human primary liver cells upon nodularin exposure. A: Huh7 cells were exposed to 0.1 (light blue bars), 1 (blue bars) and 5 μ M nodularin (dark blue bars) for 24, 48 and 72 h followed by determination of mRNA levels of *BIP*, *XBP1s*, *ATF4*, *CHOP* and *GADD34*. Due to toxicity, 5 μ M nodularin exposure was not done at 72 h. Shown are results of three independent experiments. B: Primary liver cells exposed to 1 (green bars) and 2.5 nM nodularin (blue bars) for 24 h followed by determination of mRNA levels of *BIP*, *XBP1s*, and *GADD34*. Shown are the results of two (1 nM) or three (2.5 nM) independent experiments. C: Huh7 cells exposed to 5 μ M nodularin for 24 h followed by Western blot analysis for CHOP and BiP. As loading control Western blot membrane was incubated with Actin antibody. Significant differences with p-value of ≤ 0.05 marked with asterisks.

Figure 5

Co-exposure of Huh7 cells to nodularin and the TNF- α inhibitor sauchinone. A: Huh7 cells were exposed to 30 μ M sauchinone alone (yellow bars), 5 μ M nodularin alone (green bars) and a combination of 30 μ M sauchinone and 5 μ M nodularin (blue bars) for 24 and 48 h followed by determination of mRNA levels of *TNF- α* and *IL8*. B: Huh7 cells were exposed to 5 μ M nodularin alone (green bars), or to a combination of 30 μ M sauchinone and 5 μ M nodularin (blue bars) for 24 h (horizontal strips), and 48 h (vertical strips) followed by measuring TNF- α protein level in the supernatant. Shown are results of three independent experiments. Significant differences with p-value of ≤ 0.05 marked with asterisks.

Figure 6

Molecular modes of action of nodularin as revealed in our study. In addition nodularin results in inhibition of protein phosphatases. The major effect of nodularin is induction of *TNF- α* , which is followed by downstream events, such as induction of *IL8*, *DR5* and *CASPASE 8*. Additionally, the MAPK signalling pathway is induced, mainly *CFOS* and *JUND*, resulting in the activation of the AP-1 complex. An additional but weaker effect of nodularin is the induction of ER stress and associated UPR. All of these molecular effects ultimately lead to effects in the liver.

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645 **Figure 6**

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