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Short communication

In vitro metabolism of the cyanotoxin cylindrospermopsin in HepaRG cells and liver tissue fractions

Katrin Kittler [a, b], Dominique Hurtaud-Pessel [a], Ronald Maul [b, c], Franziska Kolrep [d], Valérie Fessard [a, *]

[a] ANSES, French Agency for Food, Environmental and Occupational Health Safety, Fougères Laboratory, 10B rue Claude Bourgelat – Javené, CS 40608, 35306, Fougères Cedex, France
[b] Federal Institute for Materials Research and Testing (BAM), Richard-Willstätter-Straße 11, 12489, Berlin, Germany
[c] Leibniz-Institute of Vegetable and Ornamental Crops Großbeeren/Erfurt e.V., Theodor-Echtermeyer-Weg 1, 14979, Großbeeren, Germany
[d] Federal Institute for Risk Assessment (BfR), Max-Dohrn-Straße 8-10, 10589, Berlin, Germany

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ABSTRACT

No evidence for phase I metabolites of the cyanotoxin cylindrospermopsin (CYN) was given using HepaRG cells and different liver tissue fractions when studying metabolic conversion. Although the application of ketoconazole, a CYP3A4 inhibitor, led to a decreased cytotoxicity of CYN, no metabolites were detected applying high resolution mass spectrometry. Quantification of non-modified CYN led to recovery rates of almost 100%. Consequently, reduction of CYN toxicity in the presence of metabolism inhibiting agents must be attributed to alternative pathways.

The cyanotoxin cylindrospermopsin (CYN) has been implicated in several outbreaks of human and cattle intoxications (Griffiths and Saker, 2003; Thomas et al., 1998). The mechanisms which are involved in CYN toxicity are not fully elucidated. However, CYN has been shown to inhibit protein synthesis in various biological systems including reticulocyte lysate and primary rat hepatocytes (Froscio et al., 2001; Terao et al., 1994). In primary rodent hepatocyte cultures, CYN inhibited glutathione synthesis (Humpage et al., 2005; Runnegar et al., 1995). Additionally, CYN was shown to induce oxidative stress in vitro in several cell models (Liebel et al., 2015; Lopez-Alonso et al., 2013; Zegura et al., 2011) as well as in vivo in the liver of fish (Guzman-Guillen et al., 2013; Puerto et al., 2014); the studies on oxidative stress revealed an increased protein oxidation, lipid peroxidation and the protective role of antioxidants due to CYN.

In some studies the involvement of cytochrome P450 (CYP) enzymes in CYN associated toxicity is postulated, because CYP inhibition decreased genotoxicity and toxicity of CYN in HepaRG cells, primary mouse hepatocytes or in vivo in mice (Bazin et al., 2010; Humpage et al., 2005; Norris et al., 2002). The CYP3A4 inhibitor ketoconazole (ket), the CYP 2C19 inhibitor omeprazole as well as the unspecific CYP inhibitors piperonyl butoxide and proadifen exerted protective effects against CYN toxicity. Moreover, recently, CYP1A1 and 1A2 were shown to be up-regulated in human peripheral blood lymphocytes following CYN exposure (Straser et al., 2013; Zegura et al., 2011). i.p. treatment of mice with ^{14}C labeled CYN indicated the formation of a more polar metabolite or a catabolic degradation product; additionally a CYN protein adduct was postulated to be formed in a methanolic liver tissue precipitate (Norris et al., 2001). In a further study DNA adduct formation was detected in mouse liver after i.p. treatment with CYN containing cyanobacterial extract (Shaw et al., 2000). However, Lankoff et al. did not report an increased genotoxicity of CYN in CHO–K1 cells after metabolic activation using rat S9 (Lankoff et al., 2007). Despite the numerous investigations on the toxicity of CYN, no efforts concerning the identification of any metabolites have been published.

In the present work, CYN metabolism was investigated using rodent and human liver fractions as well as a metabolically

* Corresponding author.
E-mail address: valerie.fessard@anses.fr (V. Fessard).

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competent human hepatic cell line (HepaRG). In order to identify metabolites of CYN in vitro, a combined approach was conducted using (i) direct metabolite identification by LC-HRMS and (ii) indirect metabolism investigation by accurately quantifying the CYN depletion after in vitro incubation employing the stable isotope dilution analysis (SIDA)-LC-MS/MS method.

Initially the impact of the CYP3A4 inhibitor ket was investigated on the viability of CYN-treated HepaRG cells. Cryopreserved differentiated human HepaRG cells (Biopredic International, Rennes, France) were cultured and used for cytotoxicity studies according to (Kittler et al., 2014a). An aqueous 4 mM CYN stock solution provided by the group of Dr. A.R. Humpage was used for cytotoxicity testing in a CYN concentration range between 0.11 μM and 28.28 μM. The 50% inhibition concentration (IC_{50}) of CYN was determined after 24 h of exposure with and without ket co-incubation (10 μM; Sigma, Saint-Quentin Fallavier, France) by measuring several cytotoxicity endpoints (mitochondrial activity, lysosomal activity and total protein content) with a test kit XTT/NR/SRB (Aniara, West Chester, Ohio).

HepaRG cells possess a wide range of metabolic enzymes inferring a high metabolic competency which is considered to be comparable to human liver (Aninat et al., 2006; Antherieu et al., 2010; Le Vee et al., 2006). The CYP inhibitor ket was shown to decrease key CYP450 activities. Therefore, the involvement of CYPs in the formation of metabolites can be investigated in HepaRG cell cultures. Potential metabolites were analyzed by LC-MS in cell media (with/without ket) comparing CYN treatment and vehicle control. For sample preparation, acetonitrile (ACN) equal to the volume of the incubation assay was added to cell media. The media were centrifuged at 14,000 × g, the supernatant was evaporated to dryness and reconstituted in water prior to LC-MS analysis.

Direct detection of metabolites is challenging when i) highly reactive metabolites are formed, ii) the substrate undergoes less common or unknown reactions, or iii) only low amounts of metabolites are formed. The sensitive and accurate LC-MS/MS quantification of non-metabolized CYN using SIDA is a possibility to circumvent these obstacles. Detecting a significant decrease of CYN in HepaRG medium after CYN treatment would suggest that metabolism had occurred. HepaRG cells were incubated in the presence of 72 nM CYN for 24 h in 12 well plates treated with/without ket and CYN was quantified by SIDA-LC-MS/MS. The medium and the corresponding HepaRG cell lysate (cell disruption induced by trypsin, methanol and ultrasonication) were combined and ACN containing uniformly ^{15}N labeled CYN was added. After centrifugation the supernatant was evaporated to dryness and reconstituted with water.

Conventional liver tissue fractions i.e. induced rat S9 or human S9 as well as human microsomes were also used as model systems to elucidate CYN metabolism because of their enhanced metabolic conversion rates compared to the liver cell culture assay. CYN concentrations were measured with SIDA-LC-MS/MS in samples that were incubated in the presence of 72 nM CYN for 3 h at 37 °C using heat inactivated S9 or microsomes as reference. Phase I reactions were conducted as described in Kittler et al., 2010 for induced rat and human S9 (protein content of 2.5 mg/mL each) and according to Rüfer et al., 2007 for human microsomes studies.

As a positive control for phase I metabolic conversion, okadaic acid (OA), a known substrate of CYP3A4/A5 (Guo et al., 2010), was additionally included during the in vitro studies with HepaRG cells and during the liver tissue fraction assays at 885 nM and 100 nM, respectively.

All samples were analyzed using a Thermo Fisher Accela LC (Thermo Fisher, Bremen, Germany) system hyphenated to an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher, Bremen, Germany). CYN and its potential metabolites were separated on a Nucleodur C18 Polartec column (3 μm, 150 × 2 mm) with guard protection (Machery-Nagel, Düren, Germany). Eluent A was water and eluent B was ACN both containing 5 mM ammonium acetate. The gradient started at 100% A for 2 min, followed by decreasing A to 64% within 4.5 min and then to 20% within 2 min, then holding A for 3 min and increasing A within 2 min to initial conditions followed by equilibrating the column for 12 min. The flow rate was constant at 200 μL/min.

For metabolite screening, positive ion LC-MS analysis of the samples was conducted in high resolution full scan mode (*m/z* 100–2000; resolution: 60,000) and in parallel in low resolution ion trap MS/MS mode (CID, CE 30 V) for data dependent analysis using a parent mass list and dynamic exclusion. The parent mass list was calculated based on known mass shifts arising from typical phase I reactions using MetWorks software. Two independent MS^2 experiments were acquired in LTQ for *m/z* 416.2 and *m/z* 421.2 (CID, CE 35 V) for CYN quantification by SIDA-LC-MS/MS as described in (Kittler et al., 2014b). Xcalibur 2.0.7 was used for data handling and quantification.

A statistically significant increase of the IC_{50}, e.g. for neutral red assay from 2.7 to 4.5 μM ($p < 0.01$), was determined when co-incubating HepaRG cells with both CYN and ket (Fig. 1). Therefore, a slight protective role of ket on CYN cytotoxicity due to prevented formation of reactive CYN phase I metabolites can be assumed in HepaRG cells.

The use of HepaRG cells for metabolic conversion did not enable detecting any CYN phase I metabolites. In the LC-HRMS analyses of the media from toxicity testing no signals for target masses of potential CYN metabolites were detected in the treatment samples that were not also observed in the control samples. Nevertheless, the detection of positive control OA metabolites demonstrated the reliability of the assay (Kittler et al., 2014a).

In addition, metabolic conversion of CYN was measured by accurate quantification of remaining CYN after incubation with HepaRG cells and tissue fractions. As presented in Table 1, no decrease of CYN was reported following HepaRG cell incubations irrespective of ket co-incubation. Similarly, using different tissue fractions for metabolic conversion, a complete recovery of CYN was observed. By contrast, a significant decrease of OA concentration occurred in treated samples compared to control samples (Table 1).

Although a statistically significant shift of cytotoxicity was induced in HepaRG cells following a co-incubation with CYN and ket, no evidence for phase I metabolites of CYN could be provided applying mass spectrometric techniques. As it was shown that CYN or its metabolites could form protein adducts, such binding may mask the detection of CYN metabolites (Froscio et al., 2008). However, the quantification of the remaining parent toxin at the end of the incubation period with liver HepaRG cell line or mammalian liver tissue fractions did not indicate such irreversible binding because CYN recovery was 100% according to the applied SIDA based quantification (Table 1). Nevertheless, CYN metabolism to some extent cannot be excluded as a transformation of up to 7 nM which equals 9% of the initial CYN concentration would be within the limits of the standard deviation.

In the past, different CYPs inhibitors have been shown to protect against CYN mediated toxicity. Usually, specific metabolites are formed by one or very few distinct CYPs responsible for the conversion of a substance. The decrease of CYN cytotoxic effects in HepaRG cells may also be caused by side effects of the CYP inhibitor ket. Ket has also been shown to affect cellular uptake or efflux as already reported for various substances (Choi et al., 2011; Rautio et al., 2006; Taub et al., 2011). Therefore, ket treatment could inhibit CYN transport, leading to a lower CYN concentration inside HepaRG cells and consequently a decreased observable cytotoxicity.

In addition, ket was shown to be protective against oxidative