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Cytotoxicity evaluation of large cyanobacterial strain set using selected human and murine *in vitro* cell models

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ABSTRACT

The production of cytotoxic molecules interfering with mammalian cells is extensively reported in cyanobacteria. These compounds may have a use in pharmacological applications; however, their potential toxicity needs to be considered. We performed cytotoxicity tests of crude cyanobacterial extracts in six cell models in order to address the frequency of cyanobacterial cytotoxicity to human cells and the level of specificity to a particular cell line. A set of more than 100 cyanobacterial crude extracts isolated from soil habitats (mainly genera *Nostoc* and *Tolypothrix*) was tested by MTT test for *in vitro* toxicity on the hepatic and non-hepatic human cell lines HepG2 and HeLa, and three cell systems of rodent origin: Yac-1, Sp-2 and Balb/c 3T3 fibroblasts. Furthermore, a subset of the extracts was assessed for cytotoxicity against primary cultures of human hepatocytes as a model for evaluating potential hepatotoxicity. Roughly one third of cyanobacterial extracts caused cytotoxic effects (i.e. viability $< 75\%$) on human cell lines. Despite the sensitivity differences, high correlation coefficients among the inhibition values were obtained for particular cell systems. This suggests a prevailing general cytotoxic effect of extracts and their constituents. The non-transformed immortalized fibroblasts (Balb/c 3T3) and hepatic cancer line HepG2 exhibited good correlations with primary cultures of human hepatocytes. The presence of cytotoxic fractions in strongly cytotoxic extracts was confirmed by an activity-guided HPLC fractionation, and it was demonstrated that cyanobacterial cytotoxicity is caused by a mixture of components with similar hydrophobic/hydrophilic properties. The data presented here could be used in further research into *in vitro* testing based on human models for the toxicological monitoring of complex cyanobacterial samples.

1. Introduction

The toxicity of cyanobacteria is a perennial problem in water management and agriculture all over the world. Many examples of human and animal poisoning have been described in numerous case studies and reviews (Azevedo et al., 2002; Faassen et al., 2012; Giannuzzi et al. 2011; Stewart et al., 2008; Zanchett and Oliveira, 2013). Due to the massive occurrence of cyanobacteria in surface water, planktonic cyanobacteria have been intensively studied in terms of their production of secondary metabolites and toxins. In recent years, several studies have demonstrated that not only planktonic cyanobacteria, but also benthic, soil or symbiotic cyanobacteria can produce compounds potentially toxic to humans (Faasen et al., 2012; Cox et al., 2003; Hrouzek et al., 2012; Jokela et al., 2012).

In the past, the hepatotoxic peptides microcystins and nodularins (Campos and Vasconcelos, 2010; Pearson et al., 2010), neurotoxic alkaloids (Wiese et al., 2010) and the cytotoxic alkaloid cylindrospermopsin (Runnegar et al., 1995) were discovered to exhibit toxic effects on humans. For this reason, some of these compounds are strictly monitored in the drinking water of many countries worldwide. Despite widespread knowledge about these cyanobacterial toxins, more than 80% of cyanobacterial metabolites (potential toxins) remain unknown (Hrouzek et al., 2011; Welker and von Döhren, 2006; Welker et al.; 2006). The variety of chemical structures produced by cyanobacteria implies their possible interaction with a wide range of cellular targets, identifying new mechanisms of cyanobacterial toxicity. Recently several novel

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mechanisms of cyanobacterial metabolite toxicity were proposed, for example altering cell gap junctional communication (Nováková et al., 2013), cytoplasmatic membrane permeabilization (Jokela et al., 2012; Hrouzek et al., 2012; Tomek et al., 2015) or reactive oxygen species formation (Zegura et al. 2008, 2011, Poniedziałek et al., 2015).

Taking into account these facts, it is very important to develop methodologies for assessing the toxicity of cyanobacterial biomass containing a mixture of potentially toxic compounds. Since *in vivo* assays using mouse models are usually performed with pure toxins and in highly justifiable cases, several alternative bioassays including insects (Ruebhart et al., 2011), cladocerans (Okumura et al., 2007) and many other classes of organisms (Maršálek and Bláha, 2004) were also applied to detect cyanobacterial toxicity. However, for example a model organism, *Artemia salina*, often used for cyanobacterial toxicity screening (Lahti et al., 1995), has been shown to be insufficient, and many discrepancies were found between the *Artemia* and *in vitro* models using mammal cells (Hisem et al., 2011). Several studies indicated a good correlation between the results obtained by cytotoxicity testing using *in vitro* mammalian cells and *in vivo* mouse assay when crude cyanobacterial extracts were tested (Teneva et al. 2003, Teneva et al. 2013).

The use of cell lines has a lot of limitations. Primarily, *in vitro* systems do not mimic the complexity of the whole organism, and very often do not express the full spectrum of tissue-specific drug-metabolizing enzymes. One of the options for simulating complex physiological interactions *in vitro* is the whole-blood model which was successfully applied to study potential toxicity mechanisms of the cyanobacterial hepatotoxin cylindrospermopsin (Poniedziałek et al., 2014, 2015). Secondly, the cell lines can exhibit genotype instability over time in cultures, or have temporary viability (Guillouzo, 1998; Kanebratt and Andersson, 2008). Despite all their disadvantages, *in vitro* models have been successfully applied in many fields, especially in detecting potentially hepatotoxic agents and estimating human hepatotoxicity in early drug development (Xu et al., 2004).

As was mentioned above, many potentially toxic cyanobacterial metabolites are of unknown structure. Moreover, their complex mixture present in cyanobacterial biomass may cause various additive and multiplicative effects. Thus it is difficult to apply any specific test that would respond equally to all toxic compounds and provide a relevant piece of information in terms of assessing potential human or animal toxicity.

In this study, a complex cytotoxicity evaluation of crude extracts obtained from soil cyanobacterial strains is presented. For this purpose we selected non-transformed immortalized cells (Balb/c 3T3), routinely used in toxicological studies, and compared their responses with two human cancer cell lines (HeLa and HepG2) and two murine models, YAC-1 and Sp-2. Since the liver is a primary target organ for many toxins, we used primary cultures of human hepatocytes (HH), containing the full spectrum of biotransformation enzymes, in contrast to the limited biotransformation abilities of a HepG2 cell line, as a model for potential hepatotoxicity evaluation. We compared the response of these six cell *in vitro* systems using one common cytotoxicity endpoint measurement, the MTT test. Our results suggest that soil cyanobacterial crude extracts and their constituents exhibit mostly general cytotoxic effects resulting in highly correlated inhibition values among HeLa, HepG2 and Balb/c 3T3 cells. Moreover, a good correlations between the results obtained on primary human liver cells and Balb/c 3T3 cells and the HepG2 cell line were found.

2. Experimental section

2.1. Experimental design

The cytotoxicity study presented here is based on a comparison (correlation) of the viability of six cell systems after their exposure to crude cyanobacterial extracts using the MTT test. IC_{50} values were determined for selected cytotoxic crude extracts to compare their effectivity in HeLa, Sp-2 and Balb/c 3T3. To ensure that the effect is not caused by the whole crude extracts and that cytotoxic substituents are present, activity-guided fractionation was performed in selected strains. The results obtained with the isolated fractions were compared to the general cytotoxic effects of the extracts.

Two data sets were analyzed in this study. In the first, the cytotoxic activity of more than 100 crude cyanobacterial extracts against human (HeLa and HepG2) and murine (Yac-1, Sp-2) cancer cell lines were compared. In the second set, 50 selected cyanobacterial extracts were tested for cytotoxic activity against murine Balb/c 3T3 fibroblasts and a primary culture of human hepatocytes (HH), used as a model for potential hepatotoxicity evaluation in humans (Guillouzo, 1998; Godoy et al., 2013). The cytotoxicity data were presented as the viability of particular cell lines and the primary culture after 24 h of exposure to cyanobacterial extracts at a concentration of 10 mg d.w. (lyophilized cyanobacterial biomass)/mL (cultivation medium). The viability results were grouped into three categories in order to estimate the cytotoxic effect of particular extracts. The following ranges were used: 0-50% cell viability indicated a strong cytotoxicity of the extract (++), 51–75% viability – moderate cytotoxicity (+), viability higher than 75% – slight or no cytotoxic effect.

2.2. Origin and cultivation of cyanobacterial strains

The cytotoxicity of cyanobacterial strains collected from natural habitats and housed in the Institute of Microbiology CAS in Trebon (*Nostoc*, *Desmonostoc*, *Leptolyngbya*, *Phormidium*, *Cylindrospermum*, *Anabaena*, *Tolypothrix*, *Wollea*, *Synechocystis*, *Oscillatoria*, *Calothrix*, *Fisherella*) was tested. For the complete list of strains and their origins, see Supplementary Table S1.

The cyanobacterial strains were cultivated in 350 mL glass tubes on liquid Allen Arnon medium (Allen and Arnon 1955) and bubbled with 2% CO_2-enriched air at a constant temperature of 28 °C, under a continuous illumination of 50 W/m^2. After 5–7 days of cultivation, the culture was harvested by centrifugation (1950 × *g*, 10 min), stored at −70 °C and finally lyophilized.

2.3. Extract preparation

200 mg of lyophilized cyanobacterial biomass was extracted with 6 mL of 70% aqueous methanol using a mortar and pestle, with the addition of marine sand. The homogenates were transferred into 10 mL glass tubes and left for 1 h. The homogenates were then centrifuged (1950 × *g*, 15 min) and the supernatants transferred into 50 mL heart-shaped flasks and evaporated to dryness under reduced pressure at 40 °C. The dry residues were dissolved in 1 mL of 100% MeOH (final concentration of the crude extract: 200 mg d.w. (cyanobacterial biomass)/mL) using an ultrasonic bath, transferred into 1.5 mL Eppendorf tubes and centrifuged for 5 min at 5040 × *g*. The supernatants were transferred into 1.5 mL glass vials, analyzed by HPLC-MS and stored at −20 °C prior to cytotoxicity testing.

2.4. Activity-guided fractionation of selected extracts with high cytotoxicity

Prior to activity-guided fractionation, the extracts were