ORIGINAL ARTICLE

# Evaluation of genotoxic potential of neurotoxin anatoxin-a with the use of umuC test

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## Abstract

**OBJECTIVE:** The aim of this study was to evaluate genotoxicity of anatoxin-a, cyanotoxin of neurotoxic activity. Additionally, other frequently detected cyanotoxin of previously described genotoxic potential, microcystin-LR, was used at the same concentrations, as well as the mixture of both toxins, anatoxin-a and microcystin-LR.

**DESIGN:** Genotoxicity of the toxins was determined with the use of the umuC assay, in which the induction and expression of the umuC – lacZ reporter gene was assessed. The test was conducted on *Salmonella typhimurium* TA 1535/pSK1002 strain, with and without metabolic transformation. The toxin concentrations were 0.25, 0.5, 1 and 2 μg/ml. The exposure time was 2 h.

**RESULTS:** The highest inefficient concentration of anatoxin-a without metabolic transformation was 0.25 μg/ml, of microcystin-LR was 0.5 μg/ml and in case of the toxin mixture all used concentrations induced the umuC gene. When S9 fraction was added to the samples, no effects were detected.

**CONCLUSION:** To our knowledge, this is the first report on genotoxic effects of anatoxin-a. Although the study is preliminary and needs further research, however, indicates the new potential activity of the toxin, as well as the possible increase of genotoxicity of other cyanotoxins, more stable in the environment, e.g. microcystin-LR.

**Abbreviations:**

| | |
|---|---|
| Antx-a | - Anatoxin-a |
| MC-LR | - Microcystin-LR |
| nAChRs | - Nicotinic acetylcholine receptors |
| ONPG | - Ortho-Nitrophenyl-β-galactosidase |
| G | - Growth factor |
| $I_R$ | - Induction ratio |
| $U_s$ | - Relative β-galactosidase activity |

## INTRODUCTION

Antx-a belongs to the one of the commonly worldwide detected cyanotoxins (Pawlik-Skowrońska *et al.* 2004; Viaggiu *et al.* 2004; Wood *et al.* 2005; Hedman *et al.* 2007). The toxin is produced by several cyanobacterial genera, such as *Anabaena, Aphanizomenon, Cylindrospermum, Microcystis, Planktothrix, Nostoc* or *Oscillatoria* (Osswald *et al.* 2007a). It is a bicyclic secondary amine of low molecular weight (165 Da), with limited persistence in environment, as it undergoes fast degradation, i.a. through photochemical decomposition in sunlight (Stevens & Krieger 1991; Chorus & Bartram 1999). A half-life of the toxin persistence under normal pH conditions is estimated on 5 days (Smith & Sutton 1993).

Antx-a in vertebrates shows well documented neurotoxic activity, extensively summarized by Osswald *et al.* (2007). It acts as an agonist of nAChRs in neuronal and neuromuscular junctions, where is not hydrolyzed by acetylcholinesterase (Aronstam & Witkop 1981; Thomas *et al.* 1993). It also binds to presynaptic nicotinic receptors causing neurotransmitter release (Gordon *et al.* 1992; Wonnacott *et al.* 2000). Its final result in acute intoxication consists of respiratory arresting and finally the death. In mice the $LD_{50}$ for Antx-a in intraperitoneal application reached 250 µg/kg body weight (Devlin *et al.* 1977).

In contrast to the other frequently detected cyanotoxins, e.g. hepatotoxic MC-LR, data on toxicokinetics of Antx-a are very limited. It is known, that the toxin is absorbed quickly from the gastrointestinal tract, as the toxic effects occur rapidly after exposure (Gupta 2007). No data on toxin distribution, metabolism or elimination have been found. The information on other than neurotoxic effects of Antx-a are very few. The toxin-induced cytotoxicity, characterized by loss of viability, lactate dehydrogenase leakage, loss of mitochondrial function and DNA fragmentation, was observed after *in vitro* rat thymocytes and monkey kidney cells exposure (Rao *et al.* 2002). Apoptotic effects in thymocytes were connected with increased reactive oxygen species production and caspase activation. It was also reported, that Antx-a induced cytotoxic effects on T and B subpopulations of mouse lymphocytes (Teneva *et al.* 2005). In *Cyprinus carpio* lymphocytes suppression of proliferation, as well as apoptosis induction was observed (Rymuszka *et al.* 2008). The mechanisms of these effects are however not clear.

To our knowledge, no cancer or genotoxicity studies have been performed neither with the use of pure Antx-a, nor with Antx-a-containing environmental extracts.

One of the basic assays for genotoxicity detection is the short term bacterial umuC assay. The test detects induction of the SOS genes in the response to different kinds of genotoxic lesions in the DNA (Oda *et al.* 1985). In the SOS response, the presence of single-stranded DNA activates the RecA protein that interacts with the LexA repressor of SOS box, causing its self-cleavage from the operator. Activation of SOS genes results in DNA repair. The modified strain of *Salmonella typhimurium* TA1535/pSK1002 used in the test was derived by fusing the *lacZ* gene for β-galactosidase with *umuC*, one of the SOS genes. Recording of *umuC* induction occurs via enzyme activity measurement. Proteins encoded by *umuCD* genes are responsible for the error-prone replication of damaged DNA, leading to SOS mutagenesis (Biran *et al.* 2009).

The aim of the study was to evaluate the genotoxic effects of Antx-a, as well to compare these effects with those obtained for MC-LR and the mixture of Antx-a and MC-LR. MC-LR was chosen because there are some reports confirming genotoxicity of the toxin (Ding *et al.* 1999; Mankiewicz *et al.* 2002).

## MATERIALS AND METHODS

Antx-a in the form of (±)-anatoxin-a fumarate ($C_{10}H_{15}NO.C_4H_4O_4$) purchased from Tocris Bioscience, UK, MC-LR ($C_{49}H_{74}N_{10}O_{12}$) purchased from Alexis Biochemicals, Switzerland and a mixture of Antx-a and MC-LR at concentrations of 0.25, 0.5, 1, 2 µg/ml each, were used. Antx-a was diluted to the desired concentration in a culture medium. MC-LR was prepared as a stock solution in methanol and diluted to the final concentration in the culture medium, where the highest methanol concentration not exceeded 0.5 % and had no influence on the bacteria.

The test was conducted with the use of umuC Easy CS Genotoxicity Assay kit (Xenometrics, Switzerland) according to the producer's protocol. The assay was performed in two independent repetitions. Shortly, *Salmonella typhimurium* TA1535/pSK1002 strain was grown for 12 h at 37 °C with agitation in TGA medium containing 1% of tryptone (w/v), 0.5% of NaCl (w/v), 0.2% of glucose (w/v) and ampicillin (50 mg/L). The culture growth was checked spectrophotometrically at 600 nm, then the overnight culture was diluted 1:4 with TGA medium and incubated for the next 2 h. The bacteria at exponential growth phase were added to the 96-well plate and exposed on the cyanotoxins at the used concentrations, without or with S9 fraction, for 2h. Each determination was performed in triplicates. As the positive controls (1) without S9 fraction – 4-nitroquinolone at 463 ng/ml of DMSO, and (2) with S9 fraction – 2-aminoanthracene at 1.85ng/ml of DMSO were used. Additionally, DMSO control, the negative control without cyanotoxins, as well as blank without bacteria, were performed. After 2h incubation, ten-fold dilution in TGA medium of each well content was prepared in a new plate. The new plate was read at 600 nm twice, directly after preparing and after 2 h of incubation at 37 °C, to measure the bacterial growth. For β-galactosidase activity detection, ONPG was added and the plate was measured at 420 nm. The

**Tab. 1.** The influence of anatoxin-a (Antx-a), microcystin-LR (MC-LR) and the mixture of the toxins (Antx-a + MC-LR) on growth factor (G) and relative β-galactosidase activity units (Us) values obtained in the umuC test ($\bar{x}$ ± SD, * indicates samples considered as genotoxic).

| S9 fraction | Toxin | Toxin concentration [μg/ml] | G | $U_S$ |
|---|---|---|---|---|
| **without** | Antx-a | 0.25 | 0.875 ± 0.050 | 0.686 ± 0.132 |
| | | 0.5 | 0.807 ± 0.139 | 0.778 ± 0.052 * |
| | | 1 | 0.775 ± 0.182 | 0.871 ± 0.069 * |
| | | 2 | 0.791 ± 0.168 | 0.872 ± 0.123 * |
| | MC-LR | 0.25 | 1.001 ± 0.007 | 0.575 ± 0.168 |
| | | 0.5 | 0.963 ± 0.024 | 0.607 ± 0.140 |
| | | 1 | 0.940 ± 0.019 | 0.726 ± 0.118 * |
| | | 2 | 0.972 ± 0.076 | 0.864 ± 0.130 * |
| | Antx-a + MC-LR | 0.25 | 0.798 ± 0.095 | 0.747 ± 0.105 * |
| | | 0.5 | 0.822 ± 0.107 | 0.720 ± 0.105 * |
| | | 1 | 0.890 ± 0.085 | 0.736 ± 0.110 * |
| | | 2 | 0.953 ± 0.071 | 0.787 ± 0.175 * |
| **with** | Antx-a | 0.25 | 1.171 ± 0.004 | 0.470 ± 0.101 |
| | | 0.5 | 1.166 ± 0.055 | 0.484 ± 0.101 |
| | | 1 | 1.193 ± 0.002 | 0.584 ± 0.122 |
| | | 2 | 1.247 ± 0.067 | 0.528 ± 0.022 |
| | MC-LR | 0.25 | 1.149 ± 0.043 | 0.515 ± 0.126 |
| | | 0.5 | 1.074 ± 0.057 | 0.544 ± 0.109 |
| | | 1 | 1.073 ± 0.036 | 0.653 ± 0.126 |
| | | 2 | 1.122 ± 0.081 | 0.702 ± 0.114 |
| | Antx-a + MC-LR | 0.25 | 1.180 ± 0.030 | 0.475 ± 0.103 |
| | | 0.2 | 1.132 ± 0.062 | 0.505 ± 0.053 |
| | | 1 | 1.148 ± 0.007 | 0.588 ± 0.129 |
| | | 2 | 1.153 ± 0.112 | 0.570 ± 0.103 |

following parameters: G, $U_s$ and $I_R$ were calculated as follows:

$$G = A_{600,S} - A_{600,B} / A_{600,N} - A_{600,B}$$
$$U_S = A_{420,S} - A_{420,B} / A_{600,S} - A_{600,B}$$
$$I_R = 1/G \times A_{420,S} - A_{420,B} / A_{420,N} - A_{420,B}$$

where: $A_{600,S}$ or $A_{420,S}$ is the absorbance of the sample at 600 or 420 nm; $A_{600,B}$ or $A_{420,B}$ is the absorbance of the blank at 600 or 420 nm; $A_{600,N}$ or $A_{420,N}$ is the absorbance of the negative control at 600 or 420 nm.

The test was considered to be valid, if $I_R$ for positive controls reached value ≥ 2. The results are presented as means from two independent test repetitions. The cyanotoxin concentration was considered as genotoxic, if $I_R \geq 1.5$ and $G \geq 0.5$.

## RESULTS

Table 1 and Figure 1 present the effects of the cyanotoxins on the induction of the SOS response, both in the presence and without S9 fraction. The G values in all tested samples exceeded 0.5, which excludes cytotoxic activity of the toxins towards used bacterial strain. In the samples without metabolic activation, the highest inefficient concentration of Antx-a was found to be 0.25 μg/ml and the obtained $I_R$ values were higher comparing with those calculated for MC-LR at the same concentration or for the mixture of toxins. The highest inefficient concentration of MC-LR was 0.5 μg/ml. For the mixture containing both the toxins, the highest inefficient concentration was not established, as

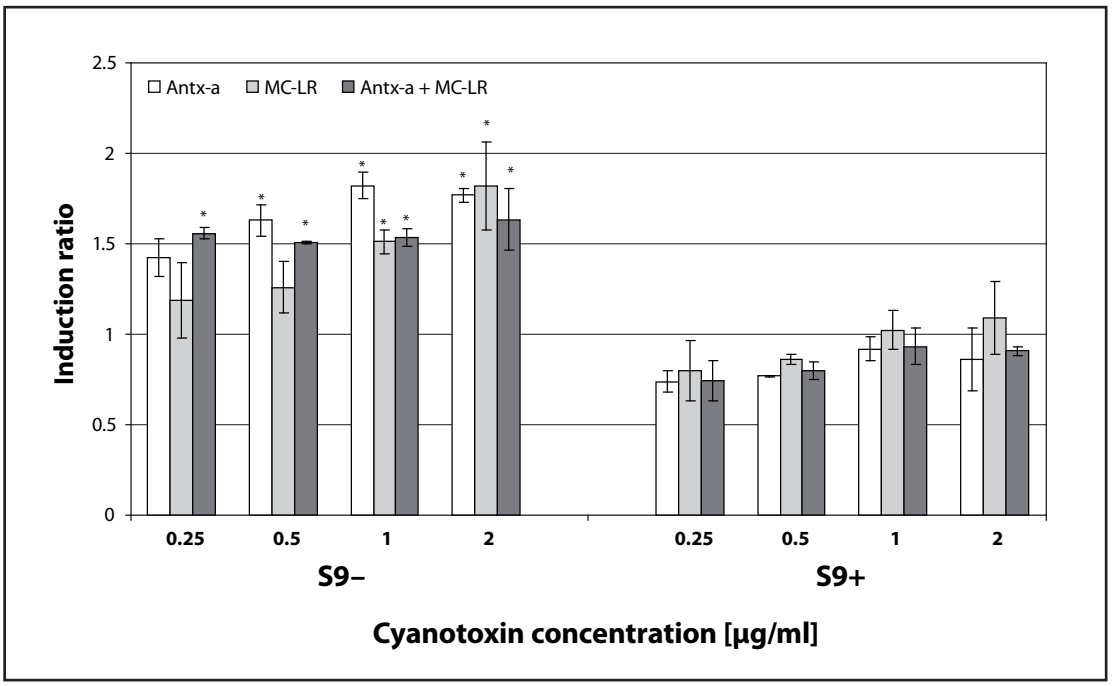


**Fig. 1.** The values of induction ratio ($I_R$) obtained after *Salmonella typhimurium* TA1535/pSK1002 exposure on anatoxin-a (Antx-a), microcystin-LR (MC-LR) and the mixture of the toxins (Antx-a + MC-LR) without (S9-) or with (S9+) metabolic transformation ($\bar{x}$ ± SD, * indicates samples with $I_R \geq 1.5$ and considered as genotoxic).

all used toxin concentrations induced relatively high β-galactosidase activity with $I_R$ values exceeding 1.5, which indicates on their genotoxic activity.

When S9 fraction was added, $I_R$ values were below 1.5 and similar to the level obtained with the negative controls, so the toxins at used concentrations after metabolic transformation were considered as non genotoxic in the conducted assay.

## DISCUSSION

Due to the fact that the umuC assay is based on the SOS system induction, it is highly sensitive in detecting DNA damage. From that reason it had been chosen as the preliminary assay to assess possible genotoxic activity of Antx-a and comparing it with MC-LR-induced effects. The obtained results show, that both the toxins possess genotoxic potential, with Antx-a being active at even lower concentration, than MC-LR.

While there are no data on genotoxicity of Antx-a in the literature, hepatotoxic MC-LR was already tested on such activity. Mankiewicz *et al.* (2002) demonstrated genotoxic effects of both microcystin-containing cyanobacterial extracts and standard MC-LR with the use of SOS Chromotest with *Escherichia coli* PQ37. The authors reported that the cyanobacterial extracts were more genotoxic, than standard MC-LR, which was the most probably related to the additional presence of other than MC-LR variants of microcystins. The positive results in SOS Chromotest were obtained only when S9 fraction was not used, which is in accordance with our findings. Moreover other tests, such as a comet assay or micronucleus test, conducted on eukaryotic cells, confirmed DNA damaging activity of MC-LR (Mankiewicz *et al.* 2002; Zhan *et al.* 2004; Huang & Xu 2009). On the contrary, there are some reports showing a lack of genotoxic or mutagenic effects of the toxin. Overview of available studies on that subject is given in Engeli (2007) and Sieroslawska (2010). Described MC-LR-induced DNA damage was linked with oxidative stress, which is one of the consequences of the toxin influence on the cells (Gehringer 2004; Engeli 2007 and references therein; Vasillakaki & Pflugmacher 2008; Wei *et al.* 2008).

At this stage, it is difficult to speculate on the mechanisms underlying observed in our study effects of Antx-a. However, the toxin, besides its ability to act as an acetylcholine agonist, was also reported to increase the oxygen reactive species production in exposed cells (Rao *et al.* 2002). That phenomenon could be considered during further research on Antx-a genotoxic activity, e.g. using the DNA-repair assay performed on repair-deficient strains of algae and mammalian cell lines with different antioxidant enzyme levels (Miadoková *et al.*, 2003; Ejchart 2001).

The next issue that should be taken into account is that in the environment different kinds of cyanotoxins are often being produced simultaneously (Osswald *et al.* 2007a). As there are indications, demonstrating genotoxic potency of MC-LR (Mankiewicz *et al.* 2002), and possibility of synergistic toxicity between Antx-a and MC-LR (Fitzgeorge *et al.* 1994), our next goal was to find out, if the mixture of these two toxins exerts different effects in the umuC assay, than each toxin alone. The obtained results were quite surprising, because while the genotoxicity was observed at each used mixture concentration, which indicates its stronger activity, the recorded $I_R$ values of the mixture, although considered as effective, were not as high as corresponding $I_R$ values of each toxin used alone, in the range when they were genotoxic.

To our knowledge, this is the first study on genotoxicity of Antx-a. That lack of more extensive research on different aspects of the toxin toxicity, probably comes from the reason, that Antx-a has a relatively short persistence in water (Osswald *et al.* 2007a;b; Billam 2006), and in case of intoxication, its neurotoxic effects are predominant. However, also other toxin activities, especially of genotoxic nature, should be carefully studied.

The results obtained in this study are preliminary and should be supported by further research, with the use of other tests, also on eukaryotic cells, e.g. yeast, algae and mammalian cell cultures (Miadoková *et al.*, 2010). However, they indicate the need to complete currently existing gap in genotoxicity estimation of Antx-a, as well as other cyanotoxins.

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