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Microcystin-LR activates the ERK1/2 kinases and stimulates the proliferation of the monkey kidney-derived cell line Vero-E6

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ABSTRACT

Microcystin-LR (MCLR) is a peptide produced by freshwater cyanobacteria that induces severe hepatotoxicity in humans and animals. MCLR is also a potent tumour promoter and it has been proposed that this activity is mediated by the inhibition of protein phosphatases PP1/PP2A, possibly through the activation of proto-oncogenes *c-jun*, *c-fos* and *c-myc*. However, the mechanisms underlying MCLR-induced tumour promotion are still largely unknown, particularly in non-liver cells. In previous studies we have demonstrated that micromolar concentrations of MCLR induce cytotoxic effects in the kidney Vero-E6 cell line. The purpose of the present work was to evaluate whether the exposure to subcytotoxic concentrations of MCLR was sufficient to induce the proliferation of Vero-E6 cells. Through BrdU incorporation assay we show that at nanomolar concentrations MCLR stimulates cell cycle progression in Vero-E6 kidney cell line. Moreover, the analysis of mitogen-activated protein kinases p38, JNK and ERK1/2 activity revealed that the proliferative effect of MCLR is associated with the activation of the pro-proliferative ERK1/2 pathway. These results emphasise the importance to confirm *in vivo* the impact of MCLR on tumour promotion at kidney level.

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1. Introduction

Microcystins (MC) are secondary metabolites produced by freshwater cyanobacteria that have been associated with severe episodes of human and animal acute hepatotoxicity (Codd et al., 2005; van Apeldoorn et al., 2007). MC are a group of approximately 70 structural variants sharing the common cyclic heptapeptide structure cyclo(-D-Ala^1-L-X^2-MeAsp^3-L-Z^4-Adda^5-D-Glu^6-*N*-Mdha^7) where X and Z are variable L-amino acids. MeAsp, Mdha and Adda are abbreviations of methylaspartic acid, methyldehydroalanine and 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, respectively (Chen et al., 2006). MCLR (with leucine-L- and arginine-R- in the variable positions) is one of the most frequent and acutely toxic variant (Funari and Testai, 2008). MC is produced by several cyanobacteria species from distinct genera such as *Microcystis*, *Anabaena*, *Planktothrix* (van Apeldoorn et al., 2007). Under favourable environmental conditions, particularly in eutrophic water resources, those organisms proliferate massively forming dense biomass concentrations (bloom) that, during the senescence phase, can release high proportions of MC into water column/surface (van Apeldoorn et al., 2007). The ingestion of contaminated raw water or inefficiently treated drinking water is the principal route for human exposure to MC (Codd, 2000). Human intoxication can also occur through haemodialysis treatment if the water that supplies the haemodialysis unit is contaminated with toxic cyanobacteria. Although less frequent, this route is the most harmful due to the direct enter of microcystins into the blood stream (Codd, 2000).

The hepatotoxicity of MC is relatively well characterized both *in vivo* and *in vitro* models (revised in Duy et al., 2000; van Apeldoorn et al., 2007). The liver specificity of MC is due to their selective uptake by hepatocytes through the membrane transport family OAPT – Organic Anion Polypeptide Transporters – that mediates the uptake and elimination of numerous xenobiotics (Hagenbuch and Meier, 2003; Fischer et al., 2005). Inside the hepatocytes, MCLR inhibits the serine/threonine protein phosphatases 1 and 2A (Yoshizawa et al., 1990). The inhibition of PP1 and PP2A induces the hyperphosphorylation of cytoskeletal proteins, which leads to hepatocyte deformation, with the consequent collapse of liver tissue organization, liver necrosis, liver haemorrhage and death (Falconer and Yeung, 1992).

Based on animal sub-chronic toxicological data, the World Health Organization (WHO, 2008) has established a guideline for microcystins in drinking water (1 nM of $MCLR_{equiv}$). This guideline

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is a provisional preventive measure to human health, as the toxicological database is limited (WHO, 2008). Besides the liver, the effects of microcystins on other organs have been barely investigated.

In fact, although OATPs are absent, or less expressed, in most non-liver cells (Fischer et al., 2005) some of the specific MC carriers have been already identified in human kidney (OATPA, OATPB) and intestine (OATPB) (Hagenbuch and Meier, 2003). In addition, some studies described *in vivo* toxic effects of MCLR in the kidney (Nobre et al., 1999; Milutinović et al., 2002, 2003), intestine (Botha et al., 2004; Gaudin et al., 2008), brain (Maidana et al., 2006), lungs (Soares et al., 2007) and reproductive system (Ding et al., 2006).

Epidemiological data has suggested an association between human chronic exposure to low levels of microcystins in drinking water and an increase in primary hepatocellular (Yu, 1995; Ueno et al., 1996) and colorectal cancers (Zhou et al., 2002). However, confounding risk factors associated with other contaminants or organisms were not ruled out and information about the levels of exposure is very limited (IARC, 2006). Consequently, an unequivocal cause-effect relationship between human exposure to microcystins and cancer development was not yet established. Nevertheless, *in vivo* two-stage rodent carcinogenesis assays have demonstrated that MCLR induces tumour promotion in rat liver (Nishiwaki-Matsushima et al., 1992) and mouse skin (Falconer, 1991) and tumour progression in mouse colon (Humpage et al., 2000). Based on its tumour promoting activity, MCLR is presently classified by IARC (2006) as a potential carcinogen to humans (class 2B).

The mechanisms underlying the MCLR-induced tumour promotion are largely unknown. However, it was suggested that mitogen-activated protein kinases (MAPK) may be involved in the tumour promotion activity of MCLR given its ability to inhibit protein phosphatases PP1 and PP2A (Gehringer, 2004). The MAPK cascade Ras-Raf-MEK1/2-ERK1/2 has a key role in cellular proliferation and is regulated by several types of phosphatases including the serine/threonine phosphatases PP1 and PP2A (Junttila et al., 2008). PP2A, in particular, has primarily an inhibitory effect on the pathway, namely on the Ras, MEK1/2 and ERK1/2 components (Junttila et al., 2008). Thus, it could be hypothesised that by inhibiting PP2A, MCLR deregulates the ERK1/2 pathway and promotes cell proliferation.

The involvement of the ERK1/2 pathway in MCLR-mediated tumour promotion has been supported only by few studies. Li et al. (2009) demonstrated that an extract of microcystins purified from a cyanobacterial bloom induced the activation the proto-oncogenes *c-jun*, *c-fos* e *c-myc* in mouse liver, kidney and testis. In fact, Fos and Jun proteins form the composite transcription factor activating protein-1 (AP-1), a mitogen-activating transactivator important for cell proliferation (Turatti et al., 2005). It is known that active ERK1/2 accumulates in the nucleus and activates transcription factors (Elk-1 and SAP 1A) that stimulate the transcription of c-Fos (Gilley et al., 2009) and that the activation of ERK1/2 stimulates the activity of AP-1 complex. This induces expression of cyclin D and promotes cell cycle progression (Chang and Karin, 2001; Fang and Richardson, 2005; Meloche and Pouysségur, 2007). Zhu et al. (2005) have demonstrated that MCLR induces the transformation of immortalized colorectal crypt cells through the constitutive activation of AKT and MAPK (p38 and JNK) cascades. This supports the hypothesis that MCLR represents a risk of colorectal cancer because cellular transformation is an initial step of carcinogenesis (Zhu et al., 2005). They also found that MCLR activates Ras and Raf, but without further effect on ERK1/2 status. Interestingly, Komatsu et al. (2007) showed that MCLR induces the phosphorylation of ERK1/2 on the embryonic kidney HEK293 cell line. However, this effect was associated with apoptosis and not with cell survival and growth.

In previous studies we demonstrated that MCLR induces cytotoxic effects (organelle changes/damages, cell lysis, apoptosis and necrosis) on the kidney-derived Vero-E6 cell line (Alverca et al., 2009; Dias et al., 2009). These effects were observed above 5 μM, according to a dose and time- dependent manner. The purpose of the present work was to evaluate the effects of low (non-cytotoxic) levels of MCLR (0.5 nM–5 μM) on the activation status of MAP kinases and on the stimulation of cell cycle progression and proliferation in the same cell line.

2. Materials and methods

2.1. Microcystin-LR and cyanobacterial extracts

Microcystin-LR was purchased from Sigma-Aldrich (CAS Number 101043-37-2) as a white solid film (purity ⩾ 95%, by HPLC). A stock solution of MCLR (1 mM) was prepared by dissolving the toxin in MEM cell culture medium (see following section). This solution was sterilized by filtration through 0.22 μm filters and kept at −20 °C until use. This form of MCLR is thereafter named "pure MCLR". Work solutions were prepared by diluting this solution in MEM culture medium.

The cyanobacteria extracts were prepared from two *Microcystis aeruginosa* strains (LMECYA 110 and LMECYA 127) isolated in 1996 from a natural bloom at Montargil reservoir, Portugal (Valério et al., 2009a) and successfully maintained in the laboratory as monoalgal, free of eukaryotes, non-axenic cultures. The production of microcystin by those strains was fully characterized by Valério et al. (2009b). SPE-HPLC-DAD analysis of cyanobacterial extracts was made according to International Standard ISO 20179 (2005), confirming the production of MCLR by LMECYA 110 and the LMECYA 127 inability to produce microcystins. This strain was tested as a negative control of *M. aeruginosa* matrix. Cultures of both isolates were performed in 2.5 L plankton light reactors (Aqua-Medic, Bissendorf, Germany) containing Z8 medium (Skulberg and Skulberg, 1990) under continuous aeration, in a 14/10 h L/D cycle (light intensity $16 \pm 4\ \mu E\ m^{-2}\ s^{-1}$, approx.) at 20 ± 1 °C. Cells harvested during exponential growth phase were lyophilized in a freeze drier (Micromodul Y10, Savant, NY, USA) and extracted with a 75% methanol solution (10 mL per 100 mg of freeze dried material) overnight at 4 °C under magnetic stirring. The extract was further sonicated with an ultrasonic probe (Sonics Vibra-Cell CV33, Sonics & Materials Inc., CA, USA), centrifuged and evaporated at 35 °C (Buchi-R, Flawil, Switzerland) to eliminate the alcoholic fraction. The resulting aqueous extract was subjected to solid phase extraction for microcystin clean-up on Sep-PakC18 cartridges (500 mg, Millipore, Bedford, MA, USA) preconditioned with methanol and equilibrated with distilled water. The MCLR containing fraction was eluted with methanol (80%, v/v) and evaporated to dryness. The solid residue was re-suspended in 50 mM acetic acid and purified by preparative chromatography through Bio-Gel P2 (40–90 μm, Bio-Rad Inc., CA, USA) packed column (Amersham Biosciences, XK 26/40, i.d./length). The mobile phase consisted of 50 mM acetic acid and the flow rate was set at $1\ mL\ min^{-1}$ (Knauner WellChrom K-120 pumps, Germany). The elution fractions (5 mL) were collected on a fraction collector (Bio-Rad Mod. 2110, CA, USA) and analysed by HPLC-DAD according to the ISO standard method 20179 using commercially available MCLR standards (Alexis Biochemicals, CA, USA). MCLR-containing fractions of LMECYA 110 and correspondent fractions from LMECAY 127 were dried in a Speed-Vac system (AES 1000, Savant), re-suspended in MEM culture medium, sterilized through PVDF 0.22 μm syringe filters and kept at −20 °C until use. Those final extracts was further analysed by HPLC-DAD to confirm the MCLR concentration. Work solutions were prepared by diluting the purified extracts in MEM culture medium.

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2.2. Vero-E6 cell line culturing

The Vero-E6 cell line (kidney epithelial cells derived from the African green monkey – *Cercopithecus aethiops*) was obtained from the American Type Culture Collection (ATCC-CRL 1586). All media and supplements were purchased from Invitrogen (Paisley, UK). Cells were grown in 25 cm^2 flasks (Nunc, Roskilde, Denmark) in Modified Eagle Medium (MEM) supplemented with 10% FBS, 0.1 mM non-essential aminoacids and 1 mM sodium pyruvate, in a 5% CO_2 humidified incubator at 37 °C. Cells in exponential growth phase were detached from the growth surface (trypsin, 0.5%), centrifuged (300g) and the cell viability was determined by the trypan blue dye exclusion method (Philips, 1973).

2.3. Evaluation of MCLR effect on ERK1/2 activation

2.3.1. Cell exposure to cyanobacterial extracts and pure MCLR

Vero-E6 cells were seeded in 35 mm culture dishes and cultured for 24 h for cell adherence and growth. The growth medium was replaced by serial dilutions of the LMECYA 110 extract in fresh growth medium, corresponding to final concentrations of MCLR ranging from 5 to 5000 nM of MCLR. The same biomass dilutions (from 0.56 to 556 $\mu g\ mL^{-1}$) of the non-toxic LMECYA 127 extract were tested in parallel as a control of *M. aeruginosa* extract matrix. Serial dilutions of pure MCLR stock solution containing 5–5000 nM of toxin were also tested. The cells were exposed to LMECYA extracts and pure MCLR for 24 h prior to MAPK analysis by immunoblotting (ERK1/2, P38 and JNK in cells exposed to LMECYA 110; ERK1/2 in cells exposed to LMECYA 127 and pure MCLR). The negative control consisted of cells grown in fresh culture medium. The positive control of the assay consisted in cells exposed to Epidermal Growth Factor – EGF (10 $ng\ mL^{-1}$, 5 min) or anisomycin (0.25 μM, 1 h). Experiments were performed in two (*M. aeruginosa* extracts) or three (pure MCLR) independent experiments.

2.3.2. Cell lysis, SDS–PAGE and Western blot analysis of ERK1/2

After toxin exposure, cells were washed with PBS and lysed with 2× Laemmli buffer. Lysates were denaturated at 95 °C for 10 min and proteins were separated on 10% SDS–polyacrylamide gels. After electrophoresis, proteins were transferred onto PVDF (polyvinylidene difluoride) membranes (Bio-Rad) and probed with either mouse-anti-phospho-ERK1/2, anti-phospho-JNK or anti-phospho-p38 antibodies from Sigma-Aldrich (dilution 1/500, Madrid, Spain). The same membranes were stripped (200 mM NaOH, 5 min) and probed with rabbit-anti-ERK1/2 antibody (dilution 1/1000, Cone MAPK-YT, Sigma-Aldrich) and mouse-anti-α-tubulin (dilution 1/1000, Sigma-Aldrich) as loading controls. Specific binding was detected after incubation with goat-anti-rabbit or goat-anti-mouse peroxidase-conjugate antibodies (dilution 1/3000, Bio-Rad) by Enhanced ChemiLuminescence (ECL) detection.

2.4. G1/S progression assay

The effect of MCLR on cell proliferation was determined using the BrdU assay, which is based on the measurement of incorporation of 5-bromo-2-deoxyuridine during DNA synthesis. Vero-E6 cells (2×10^5) were seeded on 10 × 10 mm cover slips placed in 35 mm culture dishes and were maintained for 24 h to allow cell attachment and growth. Culture medium was then replaced by toxin exposure medium supplemented with 1% of FBS and containing 0.5, 1, 5 or 10 nM of MCLR. Controls consisted of cells in culture medium supplemented with 1% or 10% of FBS. After 23 h of exposure, 60 μM BrdU (Sigma) was added for 1 h. Cells were washed with PBS, fixed in 4% (v/v) paraformaldehyde in PBS for 30 min at 4 °C, permeabilized with 100% methanol for 10 min at room temperature and then incubated with 4 N HCl for 10 min to denature DNA. The cells were neutralized with 1 M tris, pH 8.8 and then washed 3× with PBS. Cells on the coverslips were incubated with the mouse-anti-BrdU primary antibody (Roche) (dilution 1/20), followed by goat-anti-mouse secondary antibody conjugated with Texas Red (Jackson Immunoresearch Laboratories) (dilution 1/200). Coverslips were counterstained with Dapi (1.25 μg/mL), post-fixed with PFA, mounted on glass slides with Vectashield mounting medium (Vector Laboratories, Berlingame, CA, USA) and sealed with nail polish. All preparations were observed under a Leica TCS fluorescence microscope. At least 300 randomly chosen nuclei were scored for BrdU incorporation for each sample. This procedure was repeated in three independent experiments for each toxin concentration.

2.5. Statistical analysis

Results were expressed as mean values ± standard deviation (mean ± SD). Statistical analysis was performed by Student's *t*-test. Differences were considered significant when $p < 0.05$.

3. Results

The effect of exposure to a MCLR-containing *M. aeruginosa* extract on the activation state of the canonical MAP kinases p38, JNK and ERK1/2 on Vero-E6 cells was analysed by Western-blot using phospho-specific antibodies (Fig. 1). Interestingly, while all the LMECYA 110 extract dilutions (containing 5, 50, 5000 and 5000 nM of MCLR) induced a marked increase of phosphorylated ERK1/2 (p-ERK1/2 – Fig. 1), no considerable variation was found

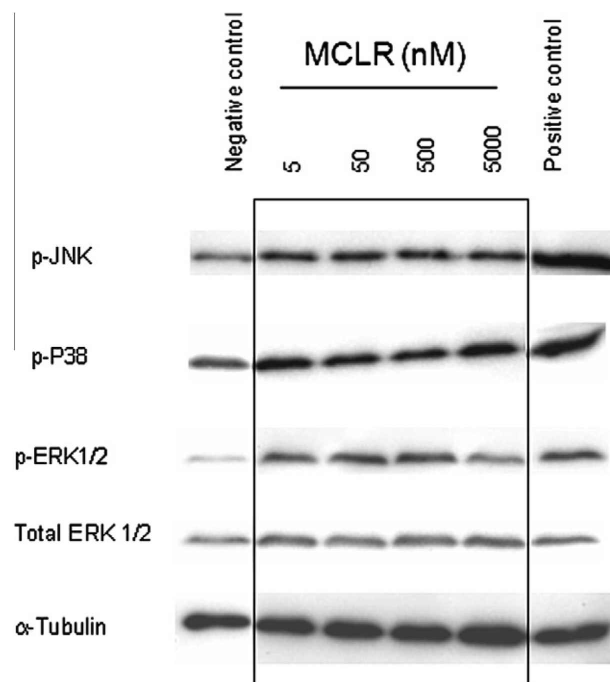

Fig. 1. Activation status of ERK1/2, p38 and JNK kinases in Vero-E6 cells by a MCLR-producer *M. aeruginosa* strain (LMECYA 110). Cells were exposed for 24 h to LMECYA 110 extract dilutions containing 5, 50, 500 and 5000 nM of MCLR. The levels of active (phosphorylated) ERK1/2, p38 and JNK, total ERK1/2 and α-tubulin (as loading control of JNK and p38) were detected by Western-blot. The negative control consisted in non-treated cells. The positive controls consisted of Epidermal Growth Factor (EGF, 10 $ng\ mL^{-1}$, 5 min) for ERK1/2 analysis and of anisomycin (0.25 μM, 1 h) for JNK and p38 analysis. Results were obtained from two independent experiments.

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in the phosphorylation state of p38 and JNK (Fig. 1). To further confirm that the observed results on ERK1/2 activity were due to MCLR exposure and not to an unknown contaminant present in the LMECYA 110 extract, the experiment was repeated using equivalent dilutions of the LMECYA 127 extract and similar concentrations of commercially available MCLR. Densitometric analysis of Western-blots from independent experiments with *M. aeruginosa* extracts revealed that whereas exposure to LMECYA 110 extract containing low concentrations of MCLR was sufficient to induce a 1.71–3.66-fold increase in p-ERK1/2, with maximum effect at 50 nM, none of the dilutions of the LMECYA 127 extract produced an effect on the activation state of ERK1/2 (Fig. 2A). A significant difference ($p = 0.037$) was found between the dose-response curves from LMECYA 110 and LMECYA 127 extracts, witch confirms the inability of LMECYA 127 to activate ERK1/2. As shown in Fig. 2B, the densitometric analysis of Western-blots from three independent experiments revealed a slightly higher overall effect in p-ERK1/2 for pure MCLR (2.8–4-fold increase). Moreover, the highest level of ERK1/2 activation was achieved at a lower toxin concentration (5 nM) then with LMECYA 110 extract. We then chose a range of pure MCLR concentrations (0.5, 1, 5 and 10 nM) around the one with highest ERK1/2 activation (5 nM) and evaluated its effect on cell cycle progression of Vero-E6 cells by the G1/S transition BrdU assay. As shown in Fig. 3, exposure of starved cells (1% FBS) to a range of 1–10 nM of MCLR clearly promoted BrdU incorporation (red cells) when compared to untreated starved cells (negative control). Quantification of the observed variations revealed a statistically significant stimulation of G1/S progression at all three concentrations, with a maximum 2.2-fold increase at 5 nM of MCLR (Fig. 4).

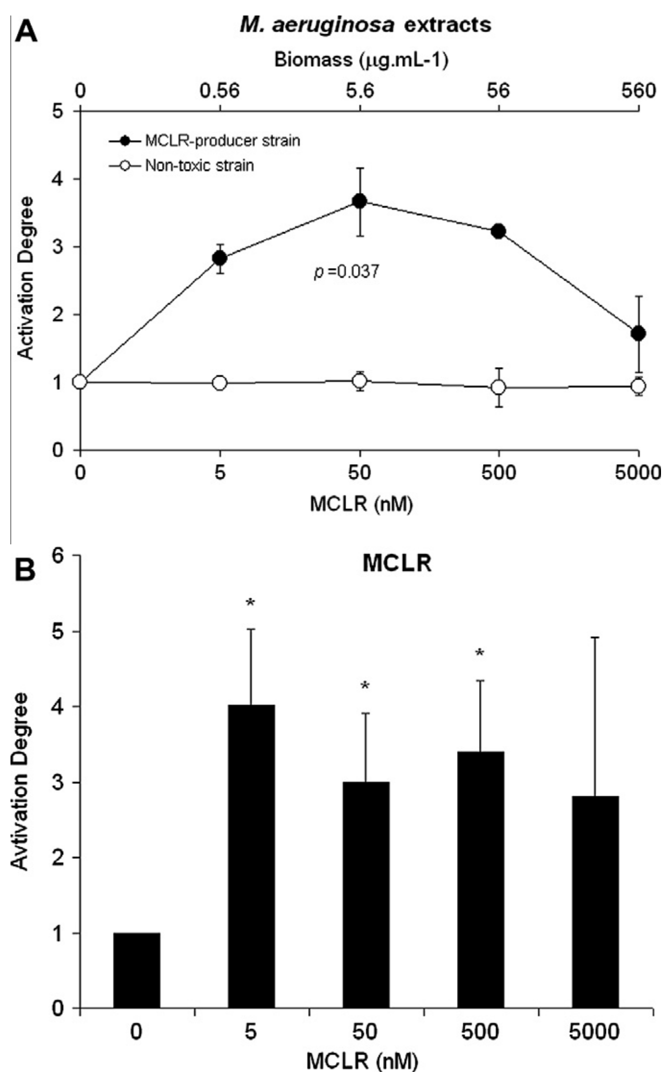

Fig. 2. Activation of ERK1/2 in Vero-E6 cells exposed for 24 h to (A) *M. aeruginosa* extracts LMECYA 110 (MCLR-producer strain) and LMECYA 127 (non-toxic strain); (B) pure MCLR. The results were obtained by densitometry analysis of Western blots and are presented as mean ± standard deviation of treated cells in relation to negative control. In Fig. 2A, secondary *x*-axis represents the *M. aeruginosa* biomass concentration and *p* indicates the significance of the difference between the two dose-response curves by a paired Student's *t*-test. In Fig. 2B, *represents a statistically significant difference between treated and control cells ($p < 0.05$).

4. Discussion

In this work we demonstrated that MCLR stimulates the proliferation of kidney-derived Vero-E6 cell line in the concentration range of 1–10 nM. We showed that both pure toxin and an extract obtained from a MCLR-producer *M. aeruginosa* strain had no effect on the activation of p38 and JNK kinases but significantly increased ERK1/2 phosphorylation with maximum effects in the 5 and 50 nM dose-range, respectively. This indicates that the stimulatory activity of low MCLR concentrations on Vero-E6 cell proliferation is mediated by the activation of this MAPK pathway.

MCLR is a potent inhibitor of protein phosphatases PP1 and PP2A (Yoshizawa et al., 1990). The key role of PP1 and PP2A in the regulation of cell division through the modulation of phosphorylation/dephosphorylation of signalling pathways have suggested that the MCLR-induced tumour promotion is mediated by MAPK (Gehringer, 2004).

Up to now, only few reports described the role of MAPK on MCLR-mediated toxicity. A study from Zhu et al. (2005) demonstrated that MCLR (0.1 nM) is able to transform immortalized colorectal crypt cells (NCC), rendering them anchorage independent and highly proliferative through the activation of the Akt and the p38 and JNK MAPK pathways. Since the transformation of colorectal cells is an important initial step in carcinogenesis (Zhu et al., 2005), these results support the hypothesis of MCLR being a risk factor of colorectal cancer (Zhou et al., 2002). In the same study, it was also demonstrated that members of the Ras and Raf families were activated, but without further activation of ERK1/2 kinases (Zhu et al., 2005). Here we found in Vero-E6 cells that low MCLR concentration affected the ERK1/2 but not the p38 and JNK MAPK pathways. The non-activation of P38 and JNK should be further confirmed by testing more exposure conditions to MCLR (time of exposure and toxin concentration). Interestingly, Komatsu et al. (2007) also reported that MCLR induced the phosphorylation of ERK1/2 in human embryonic kidney (HEK) 293 cells. They described that the activation of ERK1/2 occurred at the same toxin concentration (50 nM) that induced apoptosis and concluded that, in this cell line, apoptosis is mediated by ERK1/2. However, the HEK293 cells studied were transfected with the human hepatocyte uptake transporters OATP1B1 and OATP1B3. Therefore, the toxicokinetics of MCLR in transfected HEK293 cells is surely quite distinct from non-OATP transfected cells, given the fact that OATP expression in immortalized cell lines is reduced or even abolished (Boaru et al., 2006). Hence, the putative higher toxin absorption and accumulation in these HEK293 cells might justify their higher sensitivity to MCLR compared with Vero-E6 cells, which presented cytotoxic effects only for MCLR concentrations in the range of µM (Dias et al., 2009). Moreover, our study revealed that ERK1/2 is activated in Vero-E6 cells within a broad range of concentrations (5 nM–5 µM) whereas cell proliferation was stimulated within the range of 1–10 nM. As previously demonstrated, MCLR induces autophagy in Vero-E6 cells exposed to 5 µM, followed by apoptosis

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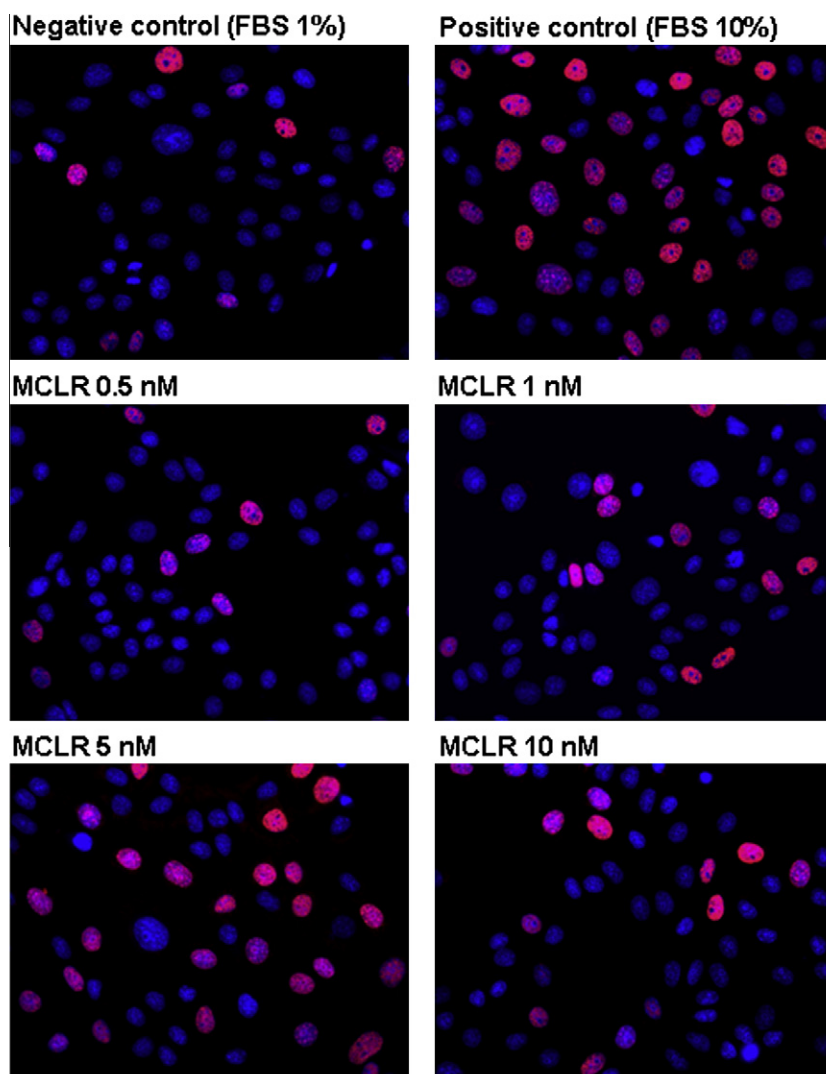

Fig. 3. Effect of pure MCLR on cell cycle progression of Vero-E6 cells. Cells were exposed to 0.5, 1, 5 and 10 nM of MCLR for 24 h under 1% FBS starvation conditions, then incubated with BrdU, fixed and stained with anti-BrdU and DAPI. Fluorescence images show BrdU incorporation into replicating nuclei in red and are representative of three independent experiments. Negative control consisted of untreated, 1% FBS starved cells. Positive control consisted of cells grown in 10% FBS-supplemented culture medium. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

above 20 μM (Alverca et al., 2009). Autophagy is a process that eliminates damaged or redundant organelles and misfolded proteins, contributing to the maintenance of cell homeostasis and survival. However, it could also function as a cell death mechanism that might collaborate with apoptotic cell death (Codogno and Meijer, 2005; Eskelinen and Saftig, 2009). Notably, the ERK1/2 pathway has been implicated in the positive regulation of autophagy (Ogier-Nenis et al., 2000; Pattingre et al., 2003; Meijer and Codogno, 2004) and in the inhibition of apoptosis (Junttila et al., 2008). Thus, we hypothesise that the MCLR-mediated ERK1/2 activation has a binary effect in Vero-E6 cells: the stimulation of cell proliferation at low MCLR concentrations and the induction of autophagic (pre-apoptotic) mechanisms at (sub)cytotoxic concentrations of MCLR. The role of ERK1/2 activation on the MCLR-mediated autophagic response as well as its eventual contribution to the inhibition of apoptosis would significantly improve the knowledge of the mechanisms underlying MCLR tumour promoting activity.

Although the main target organ of MCLR is the liver and despite most of the toxin being excreted via biliar route, a small (9%) percentage of the toxin (free or conjugated with GSH and Cys) is also eliminated through the urine (Robinson et al., 1990; Ito et al., 2002). It should be noted that although MCLR metabolites are less toxic than non-metabolized toxin, they still maintain the inhibitory activity of protein phosphatases PP1 and PP2A (Ito et al., 2002). In this way, the nephrotoxic effects of MCLR should not be disregarded, in particular those related with chronic exposure to low levels of toxin.

In conclusion, this work demonstrates that MCLR (pure or extracted from cyanobacteria) stimulates the G1/S transition of the kidney cell line Vero-E6 at very low concentrations (circa 5000 times lower than those that induce cytotoxic effects). The degree of ERK1/2 activation and subsequent G1/S cell cycle progression that was observed following MCLR treatment is comparable to that described for other tumorigenic stimuli (Moniz et al., 2007; Moniz and Peter, 2010) and thus biologically meaningful. Considering that the ERK1/2 pathway is known to be upregulated in approximately one-third of all human cancers (Dhillon et al., 2007), our results emphasise the importance to confirm *in vivo* the impact of MCLR on tumour promotion at kidney level.

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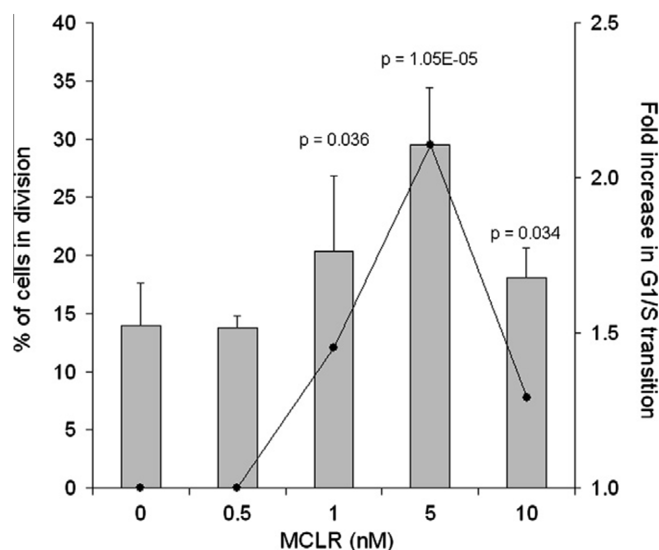

Fig. 4. Quantification of the effect of pure MCLR (0.5, 1, 5 and 10 nM) on Vero-E6 cell proliferation. BrdU incorporation was scored in at least 300 randomly chosen nuclei per sample and repeated in three independent experiments.Bars represent the mean percentage of replicating cells (±standard deviation). Line represents the induced fold increase in G1/S transition, in relation to the negative control cells. Results were obtained from three independent experiments. *p* indicates the significance of the difference between treated and control cells.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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