AVALIAÇÃO DO POTENCIAL CANCERIGÉNICO DE MICROCISTINAS (CIANOTOXINAS)

Elsa Maria Alves Dias

Doutoramento em Farmácia

(Especialidade Toxicologia)

Lisboa, 2009

AVALIAÇÃO DO POTENCIAL CANCERIGÉNICO DE MICROCISTINAS (CIANOTOXINAS)

Elsa Maria Alves Dias

Dissertação apresentada à Faculdade de Farmácia da Universidade de Lisboa para obtenção do grau de Doutor em Farmácia (especialidade de Toxicologia)

Lisboa, 2009

AVALIAÇÃO DO POTENCIAL CANCERIGÉNICO DE MICROCISTINAS (CIANOTOXINAS)

Elsa Maria Alves Dias

Tese orientada por:

Na instituição que confere o grau:

Professora Doutora Maria Camila Canteiro Batoréu
Professora Associada com Agregação da Faculdade de Farmácia da Universidade de Lisboa

Na instituição de Acolhimento:

Doutor Peter Jordan
Investigador Principal do Instituto Nacional de Saúde Dr. Ricardo Jorge

Doutora Maria João Silva
Investigadora Auxiliar do Instituto Nacional de Saúde Dr. Ricardo Jorge

O trabalho conducente à presente dissertação foi apoiado financeiramente pela Fundação para a Ciência e Tecnologia, no âmbito da bolsa de doutoramento com a referência SFRH/BD/10585/2002.

Na presente dissertação incluem-se os seguintes trabalhos publicados ou em fase de submissão para publicação:

Alverca, E., et al., Morphological and ultrastructural effects of microcystin-LR from *Microcystis aeruginosa* extract on a kidney cell line, Toxicon (2009), doi: 10.1016/j.toxicon.2009.04.014.

Dias, E., Andrade, M., Alverca, E., Pereira, P., Batoréu, M.C.C., Jordan, P., Silva, M.J. 2009. Comparative study of the cytotoxic effect of microcistin-LR and purified extracts from Microcystis aeruginosa on a kidney cell line. Toxicon 53, 487-495.

Dias, E., Pereira, P., Batoréu, M.C.C., Jordan, P., Silva, M.J. 2008. Cytotoxic and genotoxic effects of microcystins in mammalian cell lines. In: Moestrup, Ø. et al. (Eds), Proceedings of the 12th International Conference on Harmful Algae. ISSHA and IOC- UNESCO, Paris, pp. 282-285.

Dias, E., Santos, T., Pereira, P., Batoréu, M.C.C., Jordan, P., Silva, M.J. Genotoxicity of microcystin-LR on Vero-E6 and HepG2 cell lines evaluated by the Comet and the Micronucleus assays (em preparação para submissão).

Dias, E., Matos, P., Pereira, P., Batoréu, M.C.C., Silva, M.J., Jordan, P. Microcystin-LR activates the ERK1/2 kinases and stimulates the proliferation of Vero cells. (em preparação para submissão).

Para efeitos do disposto no nº 1 do Art. 40º do Regulamento de Programas de Doutoramento da Universidade de Lisboa, o autor da dissertação declara que interveio na concepção e execução do trabalho experimental, na interpretação dos resultados e na redacção dos manuscritos publicados, no prelo ou em fase de preparação para submissão.

Lisboa, 6 de Julho de 2009

(Elsa Maria Alves Dias)

Agradecimentos

A Professora Doutora Camila Batoréu foi a responsável pela minha incursão na área da Toxicologia. Foi nas suas aulas do Mestrado em "Controlo da Qualidade e Toxicologia dos Alimentos" que me contagiei com o seu entusiasmo pelas ficotoxinas. A sua generosidade marcou sempre a nossa relação orientador/orientando, quer no mestrado quer no doutoramento. Sempre me surpreendeu a sua confiança incondicional nas minhas actividades. Agradeço-lhe as suas críticas frontais e construtivas. Agradeço-lhe profundamente todo o seu empenho neste percurso académico.

Sendo investigador numa área científica tão "distante" da ecotoxicologia e não me conhecendo pessoal e profissionalmente, foi com prontidão que o Doutor Peter Jordan aceitou seu meu orientador no Instituto Nacional de Saúde Dr. Ricardo Jorge. Agradeço verdadeiramente a confiança que depositou neste projecto, a sua constante disponibilidade para com as minhas solicitações e a sua tolerância para com os meus frugais conhecimentos na área da oncobiologia.

Uma parte significativa do trabalho desta tese foi realizada sob a supervisão da Doutora Maria João Silva, que teve a tarefa difícil de me "apresentar" o mundo dos cromossomas. A morosidade inerente à aprendizagem das metodologias de Toxicologia Genética requereu uma enorme disponibilidade da sua parte, tanto mais que coordena um laboratório onde o volume de trabalho e o pessoal disponível são inversamente proporcionais. Agradeço a forma amiga e incansável com que me acompanhou ao longo deste percurso e todo o seu empenho e perfeccionismo na co-orientação deste trabalho.

O Laboratório de Microbiologia e Ecotoxicologia (LME) foi o local de iniciação às cianobactérias e cianotoxinas. Foi aqui que tive o privilégio de conhecer a Doutora Susana Franca e o Doutor Paulo Pereira. A eles devo os meus conhecimentos nesta área da Ciência, mas sobretudo os seus sábios ensinamentos para além da Ciência. Pude sempre contar com a sua profunda amizade. A amizade não se agradece, partilha-se.

No laboratório de Oncobiologia faz-se ciência com grande qualidade, empenho, entusiasmo e camaradagem. Numa área de trabalho onde não tinha experiência, fui acolhida com grande solidariedade. Por isso agradeço a todos os seus elementos. Agradeço em particular ao Paulo Matos pelo seu apoio infindável na concepção e realização do trabalho experimental, no tratamento e interpretação dos resultados e na revisão do artigo correspondente ao capítulo 6 desta tese.

Parte dos resultados apresentados nos capítulos 3, 4 e 5 desta tese resultaram da colaboração com a Telma Santos e a Mariana Andrade, no âmbito das suas teses de mestrado. A ambas agradeço a sua generosidade e empenho. A boa disposição da Telma foi particularmente contagiante.

Foi especialmente frutífera a colaboração com Elsa Alverca (co-orientadora da tese de Mariana Andrade) no âmbito do estudo dos efeitos morfológicos e ultrastururais das microcistinas. Frutífera porque constituiu uma mais valia importante para esta tese e porque permitiu estreitar laços pessoais com uma colega de longa data.

Nos 10 anos que decorreram desde a minha ida para o LME pude sempre contar com o apoio incondicional, solidário e cúmplice dos meus colegas. Quer nos aspectos profissionais, quer nos aspectos pessoais. Agradeço vivamente a: Albertina Amaral, Carina Menezes, Catarina Churro, Elisabete Valério, Filomena Sam-Bento, Ondina Pinto, Sérgio Paulino e Stela Tomé.

Agradecimento às Instituições:

Á Faculdade de Farmácia da Universidade de Lisboa, instituição que confere o grau de doutoramento a que me proponho com a presente tese. Um agradecimento particular ao Serviço de Pessoal, na pessoa de Alice Godinho, pela forma sempre tão solícita e célere com que sempre resolveu as questões burocráticas associadas a este trabalho.

Ao Instituto Nacional de Saúde Dr. Ricardo Jorge, instituição de acolhimento para a realização do trabalho conducente a esta tese.

À Fundação para a Ciência e Tecnologia, pelo financiamento do trabalho no âmbito da bolsa de doutoramento SFRH/BD/10585/2002.

Resumo

As microcistinas são metabolitos secundários produzidos por cianobactérias de água doce e constituem um risco para a saúde pública uma vez que a ingestão de água contaminada com microcistinas tem sido associada a episódios de hepatotoxicidade humana aguda e crónica.

As cianobactérias são constituintes naturais do fitoplâncton de água doce e proliferam massivamente em condições ambientais favoráveis. Porém, a pressão antropogénica sobre os recursos hídricos tem contribuído para o aumento deste fenómeno a nível global, designadamente através da contaminação das massas de água com resíduos urbanos, industriais e agrícolas, cujo conteúdo enriquecido em azoto e fosfatos constitui um estímulo para o crescimento cianobacteriano. A proliferação intensa de cianobactérias (florescência) tem como consequência a acumulação de densidades elevadas de biomassa que, após a fase de senescência, liberta para a água níveis potencialmente nocivos de cianotoxinas. Uma proporção elevada das florescências é composta por cianobacterianas tóxicas e as cianotoxinas mais frequentes são as microcistinas.

As microcistinas são um conjunto de aproximadamente 60 variantes estruturais partilhando a estrutura heptapeptídica cíclica comum ciclo(-D-alanina^{1}-L-x^{2}-D-eriro-β-iso-aspartato^{3}-L-z^{4}-Adda^{5}-D-glutamato^{6}-*N*-metil-desidroalanina^{7}) em que x e z são aminoácidos-L variáveis e Adda é o ácido (2*S*, 3*S*, 8*S*, 9*S*)-3-amino-9-metoxi-2,6,8-trimetil-10-decafenil-4,6-dienóico. A MCLR (com leucina e arginina nas posições variáveis) é a variante mais tóxica e mais comum.

O órgão-alvo principal das microcistinas é o fígado uma vez que os hepatócitos expressam ao nível da membrana citoplasmática polipéptidos transportadores dos aniões orgânicos, através dos quais as microcistinas entram na célula. Assim, a maioria dos estudos toxicológicos com microcistinas tem sido conduzida no fígado *in vivo* e em células hepáticas *in vitro*.

Com base em estudos de toxicidade aguda em animais, foi estabelecido em 1998 pela Organização Mundial de Saúde o valor-guia de 1 nM para a MCLR em água de consumo. Porém, este valor constitui uma medida preventiva parcial, uma vez que não contempla efeitos noutros órgãos nem efeitos crónicos, nomeadamente efeitos cancerigénicos. No entanto, estudos recentes têm demonstrado que a MCLR apresenta toxicidade noutros órgãos tais como os intestinos, os rins, o cérebro, pulmões e sistema reprodutor. Por outro lado, e embora a informação disponível sobre a toxicidade crónica

não permita ainda a revisão daquele valor, a MCLR está actualmente classificada pela IARC (International Agency for Research on Cancer) como um composto potencialmente cancerigénico (classe 2B).

Alguns estudos epidemiológicos associaram o aumento da incidência de hepatocarcinoma e cancro do cólon em populações humanas ao consumo de água contaminada regularmente com microcistinas. Por outro lado, estudos de carcinogenicidade em ratinhos revelaram que a MCLR é um promotor tumoral no fígado, pele e cólon. Recentemente tem sido descrita a actividade genotóxica da MCLR em diferentes tipos celulares. Contudo este é ainda um assunto alvo de alguma controvérsia na comunidade científica e não é ainda claro que a MCLR tenha, *per si*, capacidade de iniciação tumoral. Portanto, o conhecimento dos mecanismos subjacentes a uma eventual acção cancerigénica das microcistinas apresenta imensas lacunas.

O objectivo do trabalho apresentado nesta tese foi a avaliação do potencial cancerigénico de microcistinas. Numa fase inicial seleccionou-se um modelo experimental *in vitro* (trabalho apresentado no capítulo 2). Para tal avaliou-se o efeito de extractos semi-purificados de duas estirpes de *Microcystis aeruginosa*, uma produtora de MCLR e outra não produtora de cianotoxinas, no crescimento e viabilidade de linhas celulares de hepatócitos humanos (HepG2) e de ratinho (AML12) e numa linha celular de rim de macaco (Vero-E6), através de testes de citotoxicidade (MTT e LDH). A escolha dos hepatócitos é óbvia, uma vez que o fígado é o órgão-alvo das microcistinas. Usaram-se hepatócitos humanos e de ratinho porque a sensibilidade à MCLR pode depender da espécie. Usou-se também uma linha celular de rim, com o intuito, à data do planeamento do trabalho, de incluir nos ensaios um modelo celular não hepático como controlo negativo. As estirpes de *M. aeruginosa* foram isoladas de florescências naturais colhidas na albufeira de Montargil e são actualmente mantidas na colecção de algas "Estela Sousa e Silva" do Instituto Nacional de Saúde Dr. Ricardo Jorge (INSA). A caracterização da produção de cianotoxinas pelas estirpes usadas neste trabalho foi elaborada previamente no âmbito de outros trabalhos de investigação decorridos no Departamento de Saúde Ambiental do INSA. A utilização da estirpe de *M. aeruginosa* não tóxica teve como finalidade assegurar que os efeitos observados se deviam à MCLR e não a qualquer efeito da matriz do extracto cianobacteriano. Contrariamente ao esperado, a linha celular de rim Vero-E6 apresentou uma sensibilidade similar ou até ligeiramente superior à dos hepatócitos (HepG2 e AML12). Por outro lado, o extracto da estirpe produtora de MCLR induziu um efeito genotóxico (aumento da frequência de

micronucleos) nas células Vero-E6. Perante estes resultados inesperados e considerando o desconhecimento ainda existente acerca da toxicidade das microcistinas em células não hepáticas, seleccionou-se este modelo celular para a avaliação dos potenciais efeitos genotóxicos da MCLR. Para tal, a citotoxicidade da MCLR nas células Vero-E6 foi confirmada através da comparação dos efeitos de extractos de *M. aeruginosa* e MCLR pura (capítulo 3) e o limiar de citotoxicidade (25 μM) foi determinado, usando os testes MTT, LDH e *Neutral Red*. Os resultados deste trabalho demonstraram que a citotoxicidade da MCLR apresenta uma forte dependência do binómio dose/tempo de exposição e indiciaram que poderá manifestar-se primeiramente ao nível lisossomal e, sequencialmente, ao nível da mitocôndria e da membrana citoplasmática. Essa hipótese foi comprovada pelas metodologias de microscopia electrónica de transmissão e de imunofluorescência (capítulo 4). Estas metodologias permitiram identificar os alvos intracelulares da MCLR (retículo endoplasmático, lisosomas, citosqueleto, mitocôndria e membrana citoplasmática) e concluir que, de acordo com a dose e tempo de exposição, a MCLR desencadeia uma resposta autofágica nas células Vero, seguida da morte celular por apotose e necrose à medida que a dose e o tempo de exposição aumentam. Muitos destes resultados haviam sido já descritos para hepatócitos, mas apenas muito pontualmente para outros tipos celulares.

Caracterizados os efeitos citotóxicos da MCLR, foram avaliados os efeitos genotóxicos nas células Vero e nas células HepG2 (capítulo 5) através do teste do Cometa e do ensaio dos micronúcleos (MN). O primeiro permite detectar quebras na cadeia de ADN, enquanto que o segundo avalia efeitos ao nível cromossómico, designadamente efeitos resultantes da quebra de cromossomas (clastogénese) ou da perda de cromossomas (aneugénese). Os resultados obtidos comprovaram que a MCLR (em doses subcitotóxicas, 5-20 μM) induz o aumento da frequência de micronúcleos em ambas as linhas celulares, mas não induz danos na molécula de ADN. A semelhança dos resultados obtidos com as células Vero e HepG2 sugerem que a MCLR actua através de um mecanismo genotóxico comum nas células hepáticas e renais, muito possivelmente através de um mecanismo aneugénico. A distinção entre actividade clastogénica e aneugénica poderá ser importante para a avaliação do risco, uma vez que para os agentes aneugénicos pode ser possível estabelecer um limiar de exposição abaixo do qual não decorrem riscos de efeitos genotóxicos, o que não é aplicável aos agentes clastogénicos. A identificação do tipo de micronúcleos pela técnica de FISH

recorrendo a uma sonda pancentromérica permitirá esclarecer qual o mecanismo associado a este efeito genotóxico da MCLR.

Com o intuito de avaliar o efeito da MCLR na proliferação da linha celular Vero-E6, utilizou-se o teste de incorporação de BrdU, que avalia a transição G1/S do ciclo celular (capítulo 6). Os resultados permitem concluir que a exposição a doses muito baixas (1-10 nM) de MCLR estimula a proliferação das células Vero-E6. Note-se que a dose de 1nM correspondente ao valor-guia da MCLR em água de consumo definido pela OMS e está contemplado na legislação portuguesa (Dec-Lei 306/ 2007, 27 Agosto) como valor paramétrico de referência. A análise por Western-blot da expressão de cinases proteicas activadas por mitogénicos (ERK1/2, JNK, p38) revelou que a MCLR estimula a proliferação da linha celular Vero-E6 através da activação da via de sinalização ERK1/2.

Integrando os resultados apresentados nesta dissertação, poder-se-à concluir que a MCLR desencadeia uma multiplicidade de efeitos nas células Vero, sugerindo que estas poderão constituir um modelo celular adequado para o estudo dos efeitos nefrotóxicos das microcistinas. Embora o fígado seja o principal órgão de acumulação e eliminação da MCLR, cerca de 10% é excretada pela urina, pelo que os rins poderão também estar expostos a esta toxina. É de particular importância a avaliação dos efeitos decorrentes da exposição continuada a baixas doses, atendendo ao potencial cancerigénico da MCLR. Os resultados aqui apresentados acerca do efeito genotóxico e da capacidade da MCLR estimular a proliferação nas células Vero contribuem para o conhecimento dos efeitos e mecanismos subjacentes à eventual acção cancerigénica das microcistinas, sobretudo porque os estudos nesta área têm sido conduzidos maioritariamente em modelos hepáticos. Os resultados salientam, também, a necessidade de rever o valor-guia estabelecido para as microcistinas.

Abstract

Microcystins are secondary metabolites produced by freshwater cyanobacteria that constitute a risk for human health because they have been associated with acute and chronic human hepatotoxicity after the ingestion of microcystin-contaminated water.

Cyanobacteria are freshwater phytoplanktonic organisms that proliferate massively under favourable environmental conditions. However, the anthropogenic pressure on water resources has contributed to the increase of cyanobacterial proliferation worldwide, namely, through the water contamination with nitrogen- and phosphate- enriched urban, industrial and agriculture residues, that constitute a growth stimulus for cyanobacteria. The massive cyanobacterial proliferation (bloom) leads to the accumulation of high biomass densities in water that, after senescence phase, releases potential harmful levels of cianotoxins. Cyanobacterial blooms are often composed by toxic species and microcystins are the most frequent cianotoxins.

Microcystins are a group of approximately 60 structural variants sharing the common cyclic heptapeptide struture cyclo (-D-alanine^{1}-L-x^{2}-D-erythro-β-iso-aspartic acid^{3}-L-z^{4}-adda^{5}-D-Glu^{6}-*N*-methyl-dehydroalanine^{7}), where x and z are variable L-aminoacids and ADDA is (2*S*, 3*S*, 8*S*, 9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid. MCLR (with leucine and arginine in variable positions) is the most toxic and common variant.

The main target organ for microcystins is the liver because hepatocytes express the membrane organic anion polypeptide transporters; through witch microcystins enters the cell. For this reason, toxicological studies have been conducted mainly in liver *in vivo* and in cultured hepatic cells.

Based on animal acute toxicity studies, the World Health Organization has established, in 1998, the guideline of 1 nM for MCLR in drinking water. Nevertheless, this value constitutes only a partial preventive measure because it does not include the toxicity of MCLR on other organs, neither chronic effects, namely carcinogenic effects. However, recent studies have been demonstrated the MCLR induces toxicity on other organs such as the intestines, kidney, brain, lungs and reproductive system. On the other hand, despite the information on chronic toxicity of MCLR does not allow the revision of that value, MCLR is classified by the International Agency for Research on Cancer (IARC) as a potential human carcinogen (class 2B).

Some epidemiologic studies have associated the increase of human hepatocarcinoma and colorectal cancers with the ingestion of frequently microcystin-

contaminated water. On the other hand, rodent carcinogenicity studies have revealed that MCLR is a tumour promoter in liver, skin and colon. Recently, the genotoxic activity of MCLR has been described in several cell types. However, this is a matter of some controversy in scientific community and it is still not clear if MCLR can act as a tumour initiator. Thus, the mechanisms underlying an eventual carcinogenic activity of microcystins are still largely unknown.

The aim of this thesis was the evaluation of the carcinogenic potential of microcystins. The first step was the selection of an *in vitro* cell model (presented in chapter 2). For that purpose, the effects of semi-purified extract from two *Microcystis aeruginosa* strains, a MCLR-producer and a non-toxigenic strain, on the growth and viability of human (HepG2) and mouse (AML12) hepatocytes and of a monkey kidney-derived cell line (Vero-E6), were evaluated by cytotoxicity assays (MTT and LDH).The use of hepatocytes is obvious given the fact of the liver be the target-organ of microcystins. Human and mouse hepatocytes were tested because the sensibility to MCLR may be specie-dependent. The kidney cell line was used, with the initial intent, to include a non-hepatic cell line as a negative control. *M. aeruginosa* strains were isolated from natural blooms collected in Montargil reservoir and belongs, presently, to the "Estela Sousa e Silva" algal collection from National Health Institute Dr. Ricardo Jorge (INSA). The characterization of cyanotoxin production by those strains was previously performed by other researchers at the Department of Environmental Health /INSA. The non-toxigenic *M. aeruginosa* strain was used to ensure that the observed toxic effects of MCLR-producer strain were due to MCLR and not to the cyanobacterial extract matrix. Conversely to what could be expected, the kidney Vero-E6 cell line showed a similar, or even higher, sensitivity to MCLR than the hepatocyte cell lines (HepG2 and AML12). In addition, the extract from the MCLR-producer strain induced a genotoxic effect (increase of micronuclei frequency) in Vero cells. Given these unexpected results, and considering the uncertainties regarding the toxicity of microcystins in non-hepatic cells, the Vero-E6 cell model was selected to further evaluate the potential genotoxic effects of MCLR. For that purpose, the cytotoxicity of MCLR was confirmed in this cell model, by comparing the effects of *M. aeruginosa* extracts and pure MCLR (chapter 3). The threshold of cytotoxicity was determined (25 μM) by the MTT, LDH and Neutral Red cell viability assays. The results from this work have demonstrated that the cytotoxicity of MCLR strongly depends on the dose/time of exposure and showed that it is exerted sequentially on lysossomes, mitochondria and

cell membrane. This hypothesis was confirmed by transmission electron microscopy and immunofluorescence (chapter 4). These methods enabled to identify the intracellular targets of MCLR (endoplasmic reticulum, lysosomes, mitochondria and cell membrane) and to conclude that, accordingly to the dose and time of exposure, MCLR triggers an autophagic response in Vero cells, followed by apoptotic and necrotic cell death. Many of these results were previously described for hepatocytes, but rarely for other cell types.

After the characterization of the cytotoxic effects of MCLR, the genotoxic effects were evaluated on Vero and HepG2 cell lines (chapter 5) by the comet and the micronucleous (MN) assays. The Comet assay detects DNA strand breaks and the MN assay evaluates the effects at chromosome level, namely, chromosome breaks (clastogenic effect) or chromosome loss (aneugenic effect). The results demonstrate that MCLR (at subcytotoxic doses, 5-20 μM) induces the increase in MN frequency on both cell lines, but it does not induce damages in DNA molecule. The similarity of results between Vero and HepG2 cells suggests that MCLR acts through a common genotoxic mechanism in liver and kidney cells, probably by an aneugenic mechanism. The distinction between clastogenic and aneugenic activity could be important for risk assessment, because for aneugenic compounds it may be possible to establish a threshold level, below which no hazard to human health is predicted, witch is not valid for clastogens. The identification of the MN by the fluorescence in situ hybridization (FISH) technique using a pancentromeric probe will enable to clarify the mechanism underlying this genotoxic effect of MCLR.

To effect of MCLR on Vero-E6 cell line proliferation was determined by the BrdU incorporation assay that evaluates the G1/S transition in cell cycle (chapter 6). The results showed that the exposure to low doses of MCLR (1-10 nM) stimulates the proliferation of Vero-E6 cells. It should be noted that 1nM corresponds to the WHO guideline for MCLR in drinking water and is a mandatory level in Portuguese water legislation (Dec-Lei 306/ 2007, 27 Agosto). The Western-blot analysis of mitogen activated protein kinases (ERK1/2, JNK and p38) revealed that MCLR stimulates Vero cells proliferation by the activation of the ERK1/2 signalling pathway.

Taken together, the results presented in this thesis shows that MCLR triggers a multiplicity of effects on Vero-E6 cell line, suggesting that this cells might be an appropriate cell model to study the nephrotoxic effects of MCLR. Although the main target organ for MCLR accumulation and elimination is the liver, approximately 10 %

is excreted by the urine, witch means that the kidney might also be exposed. Given the potential cancerigenic effects of MCLR, it is of major importance to evaluate the effects of prolonged exposure to low doses. The results presented here regarding the genotoxic activity of MCLR and its ability to stimulate the proliferation of Vero cells contributes to the knowledge of the effects and mechanisms underlying the eventual cancerigenic activity of microcystins, primarily because the studies in this area have been conducted mainly in hepatic models. The results also emphasise the importance to re-evaluate the guideline of microcystins.

Lista de abreviaturas

ABS, absorvância**;**

ADDA, ácido 3-amino-9-metoxi-2,6,8-trimetil-10-decafenil-4,6-dienóico;

ADME, absorção, distribuição, metabolização, eliminação;

ADN, ácido desoxirribunucleíco;

BrdU, 5-bromo-2-deoxiuridina;

Cys, cisteína;

DDI, dose diária aceitável;

ELISA, *enzyme-linked immunusorbent assay*;

EMS, sulfonato de etilmetano

ERK1/2, *extracellular-signal-regulated kinase*;

FISH, hibridação *in situ* de fluorescência;

GSH, forma reduzida do glutationo;

HCC, carcinona hepatocelular;

HepG2, linha celular de hepatocarcinoma humano;

HPLC-DAD, cromatografia líquida de alta pressão com detecção por díodos;

IARC, *international agency for cancer research*;

i.p., intreperitoneal;

i.v., intravenoso;

JNK, *Jun amino-terminal kinases*;

LD_{50}, dose letal para 50% dos animais testados,

LDH, lactato desidrogenase;

LMECYA, colecção de culturas de cianobactérias "Estela Sousa e Silva" do Instituto Nacional de Saúde Dr. Ricardo Jorge;

LOAEL, dose minima que causa efeito adverso*;*

MAPK, cinases proteícas activadas por mitogéneos;

MC, microcistina(s);

MC-LA, -LR, -RR, -YR, microcistina-LA, -LR, -RR e YR, respectivamente;

Mdha, metildesidroalanina;

MeAsp, ácido metilaspártico;

MTT, brometo de 3-(4.5-dimetiltiazol-2-il)-2,5-difeniltetrazolium;

NOAEL, dose maxima que não causa efeito adverso;

NR, vermelho neutro;

OATP, transportadores polipeptídicos dos aniões orgânicos;

OMS, organização mundial da saúde;

PP1/PP2A, fosfatases proteicas do tipo 1 e 2A;

ROS, espécies reactivas de oxigénio*;*

$t_{1/2}$, tempo de semi-vida;

TUNEL, *terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling*;

Vero-E6, linha celular epitelial de rim de macaco verde africano *Cercopithecus aethiops*;

Índice de figuras e tabelas

Figuras

No capítulo 1

No capítulo 7

Tabelas

No capítulo 1

No capítulo 7

Índice

CAPÍTULO 1

INTRODUÇÃO

1.Cianobactérias e cianotoxinas

A Ecotoxicologia de cianobactérias é uma área científica que floresceu nos anos 1980-1990, quando o desenvolvimento de metodologias analíticas permitiu identificar as toxinas cianobacterianas (Sivonen e Jones, 1999). Porém, e não obstante o impacto que a ocorrência de fitoplâncton tóxico de água doce poderá ter na saúde humana e animal, e de alguns países disporem de um quadro legal que regulamenta o teor de cianobactérias e cianotoxinas (apenas microcistinas) em água para utilização humana, existem ainda muitas lacunas no conhecimento técnico e científico que impedem a avaliação inequívoca do risco de exposição humana a cianotoxinas.

Neste capítulo introdutório apresentar-se-á resumidamente o estado da arte relativamente à ocorrência de cianobactérias e toxinas associadas em reservatórios de água doce superficial e rever-se-á, com maior detalhe, o conhecimento sobre as microcistinas.

1.1. Ocorrência de florescências cianobacterianas em meio hídrico

As cianobactérias são procariontes fotossintéticos que povoam diversos habitats, sobretudo o meio dulçaquícola (Mur et al., 1999). Constituem uma classe muito diversificada de organismos, abrangendo cerca de 2000 espécies agrupadas em 150 géneros (Mur et al., 1999). Em determinadas condições ambientais favoráveis reproduzem-se massivamente, dominando toda a comunidade fitoplanctónica e formando uma densa camada de biomassa que se distribui horizontalmente, à superfície, ou verticalmente pela coluna de água. Este fenómeno designa-se habitualmente por florescência ou *bloom*.

As florescências cianobacterianas resultam da versatilidade metabólica e fisiológica das cianobactérias. As cianobactérias são organismos procariontes, unicelulares ou coloniais, que embora sejam classificadas de acordo com o código de taxonomia bacteriano, apresentam a particularidade de produzir oxigénio através da fotossíntese. Aliás, são considerados os organismos responsáveis pela introdução de oxigénio na atmosfera primitiva da terra. Têm a capacidade de adaptação cromática, ou seja, de regular o seu teor de pigmentos fotossintéticos de modo a utilizarem eficazmente o espectro da radiação. Muitas espécies têm vesículas gasosas de forma a poderem ajustar o seu posicionamento na coluna de água, de acordo com as condições de luz e temperatura mais favoráveis. Algumas espécies coloniais desenvolvem células especializadas na fixação de azoto atmosférico (heterocistos), ou que constituem formas

de resistência celular (acinetos) em condições adversas. Estas são apenas algumas das características que conferem às cianobactérias uma elevada capacidade de adaptação ao meio ambiente e, portanto, uma vantagem competitiva sobre os outros organismos fitoplanctónicos [revisto em Graham e Wilcox (2000) e Mur et al. (1999)].

A pesquisa bibliográfica sobre a ocorrência de cianobactérias revela que os *blooms* cianobacterianos apresentam uma distribuição mundial generalizada e uma maior frequência nas últimas décadas. Este aumento tem sido atribuído a causas antropogénicas (Mur et al., 1999), tais como a retenção artificial dos cursos de água, a utilização excessiva de fertilizantes, a produção animal intensiva e a contaminação dos recursos hídricos com resíduos industriais e urbanos, resultando no excessivo enriquecimento das massas de água com nutrientes e minerais (compostos azotados, fosfatos, metais, etc.). Porém, a eutrofização dos recursos hídricos não justifica totalmente a ocorrência de florescências, uma vez que estas dependem também de variações climáticas e sazonais, sendo um fenómeno natural caracterizado por alguma imprevisibilidade. De facto, mesmo em casos de reservatórios de água doce superficial sujeitos a um plano de monitorização adequado, nem sempre é possível detectar antecipadamente a ocorrência massiva de cianobactérias. Recentemente, têm surgido alguns trabalhos na área da modelação ambiental com o intuito de desenvolver modelos que permitam prever este fenómeno (Teles et al., 2006).

As florescências cianobacterianas apresentam um impacto negativo do ponto de vista ambiental. De entre outras consequências, destacam-se a turvação da água, com o consequente impedimento da passagem de luz para níveis de maior profundidade, e a desoxigenação da água, o que poderá traduzir-se no desequilibro do ecossistema, incluindo, por exemplo, a mortandade de peixes (Vasconcelos, 2006). Por outro lado, os sistemas de tratamento de água nem sempre estão preparados para a remoção de grandes quantidades de biomassa e de matéria orgânica (resultantes da decomposição das florescências), o que poderá resultar na alteração das características organolépticas da água para consumo humano (Codd, 2000). Acarretam também consequências negativas do ponto de vista económico na área da aquacultura, designadamente a mortalidade dos organismos e o decréscimo de produtividade do sistema (Smith et al., 2008). Contudo, a consequência mais preocupante associada à ocorrência de cianobactérias é a sua capacidade de produzir toxinas potencialmente nocivas para os animais e para o Homem.

1.2. Toxinas produzidas por cianobactérias

A característica mais surpreendente das cianobactérias é a sua capacidade de produzir metabolitos secundários que apresentam elevada toxicidade para outros organismos. As cianotoxinas constituem um grupo diversificado de compostos quer em termos químicos, quer em termos toxicológicos, e, sob este aspecto, podem dividir-se em toxinas com efeitos hepatotóxicos (microcistinas, nodularinas e cilindrospermopsina), neurotóxicos (anatoxina-a, anatoxina a(S), saxitoxina e derivados) e dermatotóxicos (aplysiatoxina, lyngbyatoxina, LPS) (Sivonen e Jones, 1999). Porém, é ainda uma incógnita para a comunidade científica a razão pela qual as cianobactérias produzem toxinas. Embora possam afectar organismos do zooplâncton, as cianotoxinas apresentam baixa toxicidade nos moluscos e crustáceos (pelo que estes poderão constituir vectores de toxicidade), a toxicidade nos peixes pode depender das espécies e dos seus hábitos alimentares, mas são no entanto bem conhecidos os seus efeitos tóxicos nos mamíferos (Vasconcelos, 2001a). Assim, as cianotoxinas afectam, sobretudo, organismos que não competem directamente com as cianobactérias pelo habitat, que não são seus predadores directos e que estão muito distantes na escala evolutiva.

Embora as cianotoxinas possam acumular-se em vários organismos aquáticos e propagar-se através da cadeia alimentar (Ibelings e Chorus, 2007; Zhang et al., 2009), os casos de intoxicação humana por cianotoxinas que têm sido descritos (revisto em Chorus et al., 2000; Duy et al., 2000) resultam fundamentalmente da ingestão acidental de água de recursos hídricos contaminados durante actividades balneares, ou de ingestão de água para consumo indevidamente tratada. A hemodiálise é outra via de exposição humana, em casos em que o tratamento da respectiva água de abastecimento para remoção de cianobactérias e cianotoxinas for insuficiente. Aliás, o caso mais emblemático de intoxicação humana grave ocorreu precisamente numa unidade de hemodiálise (ver 2.4). A maior parte destes registos relacionam-se com intoxicações agudas, embora estejam já descritos alguns efeitos a longo termo (cancro hepático e do cólon) em populações expostas cronicamente a água não tratada e frequentemente contaminada com cianobactérias (Yu, 1995; Ueno et al., 1996; Zhou et al, 2002) (ver 2.4).

Tal como os *blooms* cianobacterianos, também a ocorrência de cianotoxinas é extremamente difícil de prever, sobretudo porque a produção de toxinas não está confinada a determinadas espécies de cianobactérias. De facto, dentro do mesmo

género/espécie podem encontrar-se espécies/estirpes toxigénicas e não toxigénicas (Sivonen e Jones, 1999). Uma mesma estirpe pode até produzir mais do que um tipo de toxina. Por outro lado, a mesma toxina pode ser produzida por espécies pertencentes a géneros distintos. Portanto, para além de depender da densidade de organismos produtores, a ocorrência de cianotoxinas é fortemente determinada pela composição da comunidade fitopanctónica e, em última análise, pelo estado fisiológico e metabólico das espécies toxigénicas e mesmo até de condições ambientais favoráveis (Sivonen e Jones, 1999; Funari e Testai, 2008).

Até à data não é ainda possível proceder a uma correcta avaliação do risco de exposição humana a cianotoxinas. Por um lado, por insuficiência de informação científica que permita caracterizar cabalmente os respectivos mecanismos de acção e efeitos tóxicos. Por outro lado, alguns desses efeitos poder-se-ão confundir com efeitos de outros contaminantes (alimentares e hídricos) ou até mesmo com sintomas associados a determinadas patologias. Adicionalmente, e atendendo às possíveis vias de exposição humana a toxinas cianobacterianas, torna-se difícil determinar com precisão a dose a que o Homem está potencialmente sujeito. Estas limitações não permitem, portanto, estabelecer inequivocamente uma relação causa-efeito entre a exposição a cianotoxinas e a enfermidade humana.

Porém, e com base nos dados disponíveis, a Organização Mundial de Saúde (OMS) estabeleceu Níveis de Alerta relativamente à densidade de cianobactérias em água bruta na origem (Chorus et al., 2000) e um Valor Guia de carácter provisório (ver 3.3) relativo, apenas, ao teor de microcistinas na água para consumo humano (WHO, 1998). As microcistinas foram as únicas toxinas para as quais se estabeleceu um valor de segurança porque são potencialmente produzidas pelas espécies cianobacterianas predominantes nas florescências de água doce (pertencentes aos géneros *Microcystis*, *Oscillatoria (Planktothrix)*, *Anabaena* e *Nostoc*) (Sivonen e Jones, 1999). Por outro lado, as microcistinas apresentam uma elevada distribuição à escala mundial e a sua ocorrência tem sido descrita na Europa (Alemanha, Dinamarca, Eslovénia, Espanha, Finlândia, França, Holanda, Irlanda, Noruega, Portugal, Reino Unido, Rússia,), América (Brasil, Canadá, EUA), Ásia (China, Japão), Norte de África (Marrocos, unisia), África do Sul e Austrália (Sivonen e Jones, 1999; Fastner et al. 2001). Consequentemente, são as toxinas mais estudadas e sobre as quais existe mais informação toxicológica.

1.3. Ocorrência de microcistinas em Portugal

A monitorização de cianobactérias e cianotoxinas em Portugal revela-se da maior importância em termos de saúde pública, uma vez que é comum a utilização de reservatórios de água superficial eutrofizados como fonte de água para consumo humano (Vasconcelos et al., 1996). Por outro lado, a densidade cianobacteriana em águas superficiais portuguesas ultrapassa, com alguma frequência, o nível de alerta de risco moderado ou mesmo elevado estabelecidos pela OMS (Galvão et al., 2008; Valério et al., 2008, Vasconcelos, 1994). Na figura 1A exemplifica-se o aspecto que a massa de água pode apresentar durante uma florescência cianobacteriana, neste caso junto à torre de captação da albufeira do Roxo (em 2005), que fornece água para consumo da população de Beja.

Embora o estudo da ocorrência de cianobactérias em reservatórios de água doce em Portugal remonte à década de 1930, só nos anos 90 teve início o estudo da toxicidade e distribuição de espécies tóxicas com os trabalhos de Vasconcelos e colaboradores, sobretudo no centro e norte do país (revisto em Vasconcelos, 2001a).

A primeira descrição da ocorrência de cianobactérias tóxicas data de 1993, após a análise de um *bloom* dominado por *Microcystis aeruginosa* na albufeira de Crestuma (Rio Douro) em 1989 (Vasconcelos, 1993). A análise de toxinas na biomassa cianobacteriana revelou a presença de microcistina-LR (MCLR), a variante mais tóxica das microcistinas (ver 2.1). Este trabalho alertou para a necessidade de monitorizar água destinada ao consumo humano, já que se acumularam densidades elevadas de *M. aeruginosa* junto aos pontos de captação na albufeira de Crestuma que fornece água para a região do grande Porto (população de cerca de 2,000.000 habitantes).

Um estudo mais abrangente, realizado entre 1989 e 1992 em 36 sistemas de água doce (rios, lagoas e albufeiras) de norte a sul do país, confirmou que a proporção de *blooms* tóxicos é elevada (60%), nomeadamente em reservatórios destinados ao fornecimento de água para consumo humano (rios Douro, Minho e Guadiana e Lagoa de Quiaios, por exemplo) e que a espécie *M. aeruginosa* é predominante (73%) (Vasconcelos, 1994). Este estudo alertou também para o facto dos processos de tratamento à data praticados na maioria das estações de tratamento, não incluírem a filtração em carbono activado e ozonização, os únicos processos eficazes na remoção de microcistinas (Vasconcelos, 1994; Rodríguez et al., 2007).

A caracterização das microcistinas produzidas por 10 estirpes de *M. aeruginosa* isoladas a partir de reservatórios e lagoas do norte e centro do país em 1991 confirmou

que a variante MCLR é dominante, sendo produzida por todas as estirpes e correspondendo entre 44 a 100% do teor total de microcistinas (Vasconcelos et al., 1995). A variante MCLA apenas foi detectada nas estirpes isoladas de lagoas (50% das estirpes) e o seu teor variou entre 0,6-47%. A variante MCYR só foi detectada em estirpes de albufeiras (20% das estirpes) e o seu teor variou entre 11,6 e 14.7%. A variante MCRR foi detectada só numa estirpe (10%), mas numa proporção elevada (33%). Outras duas variantes foram detectadas apenas numa amostra e a sua proporção foi reduzida (MCAR, 2,8% e [D-Asp^3]MCLR, 0,8%). Estes resultados sugerem que as variantes metiladas das microcistinas (ver 2.1) são as mais comuns em Portugal (Vasconcelos et al., 1995).

A caracterização das microcistinas em amostras naturais colhidas entre 1989 e 1992 nos rios Minho e Guadiana, nas Lagoas de Mira e Barrinha de Mira e nas albufeiras de Crestuma, Torrão, Carrapatelo, Agueira e Vale das Bicas, confirmaram que, de facto, as microcistinas são as toxinas predominantes em Portugal e que a variante MCLR é a mais comum, com uma proporção, relativamente ao conteúdo total de microcistinas, de 45,5 a 99,8%, sendo também frequentes as variantes MCRR e MCYR (Vasconcelos et al., 1996).

Em 1996 foi proposto pela Direcção Geral da Saúde (DGS) o programa nacional de monitorização de cianobactérias e cianotoxinas em reservatórios de água doce que, desde então, tem sido aplicado regularmente por alguns laboratórios.

No Laboratório de Biologia e Ecotoxicologia (LBE) do Instituto Nacional de Saúde Dr. Ricardo Jorge, têm sido monitorizadas, fundamentalmente, albufeiras do centro e sul de Portugal. O acompanhamento de reservatórios de água doce do Alentejo (Alqueva, Alvito, Enxoé, Monte Novo, Odivelas e Roxo) utilizados para produção de água de consumo humano e rega, entre Maio/Dezembro de 2005 e Abril/Julho de 2006, revelou que as cianobactérias são os organismos fitoplanctónicos dominantes, ocorrendo em 52 das 53 amostras analizadas (Valério et al., 2008). Revelou, ainda, que as espécies *Microcystis spp.* são predominantes, correspondendo a 30 % da densidade total de cianobacterias, e que em duas das albufeiras foram identificados *blooms* monoespecíficos de *M. aeruginosa*. Em 23% das amostras foram detectadas microcistinas.

A partir de amostras de florescências têm-se isolado estirpes de cianobactérias que constituem parte da Colecção de Culturas de Microalgas “Estela Sousa e Silva” do

LBE[1], que, de alguma forma, espelha a diversidade de espécies de cianobactérias que ocorrem no ambiente natural. A caracterização morfológica e molecular de 95 estirpes isoladas de 21 albufeiras[2] mostrou que a espécie *M. aeruginosa* representa 44% das estirpes isoladas (Valério et al., 2009a). A análise da capacidade toxigénica dessas estirpes demonstrou que 21% produzem microcistinas, que 42% produzem mais do que uma variante simultaneamente e que as variantes detectadas são: MCLR (69%), MCRR (62%), MCYR (31%) e [D-Asp^3]MCRR (4%) (Valério et al., 2009b).

A compilação numa publicação recente (Galvão et al., 2008) dos resultados da monitorização do rio Guadiana desde 1996, de quatro albufeiras do Algarve (Bravura, Funcho, Odeleite e Beliche) desde 2001 e de cinco albufeiras do Alentejo (Alqueva, Alvito, Enxoé, Odivelas e Roxo) em 2005/2006, confirmou que cerca de 23% das amostras continham microcistinas, maioritariamente associadas à espécie *M. aeruginosa*.

A situação em Portugal, relativamente à dominância de *Microcystis spp*. e ao perfil de microcistinas, parece assemelhar-se à de outros países como a República Checa, Coreia e Japão (Vasconcelos, 1996; Chorus, 2001). Nos países do norte da Europa, como a Noruega e a Finlândia, por exemplo, as espécies dominantes e produtoras de microcistinas são sobretudo *Anabaena spp*. e as variantes desmetiladas de microcistinas (que de um modo geral são menos tóxicas - ver 2.1) são bastante comuns (Vasconcelos et al., 1996).

Saliente-se, ainda, que em Portugal a espécie *M. aeruginosa* também co-ocorre com outras espécies potencialmente produtoras de microcistinas, tais como *Anabaena spp.* (Vasconcelos, 1994; Valério et al., 2008; Galvão et al., 2008) (figura 1B) e *Planktothrix rubescens* (Valério et al., 2008; Paulino et al., 2009), bem como com espécies produtoras de neurotoxinas (Pereira et al., 2001). Em Portugal foram também já isoladas estirpes da espécie *Cylindrospermopsis raciborskii* que, no entanto, não apresentaram capacidade toxigénica (Saker et al., 2003). Porém, esta espécie tem sido descrita noutros países como produtora de cianotoxinas, nomeadamente microcistinas e cilindrospermopsina (citado em Saker et al., 2003).

[1] A colecção de microalgas "Estela Sousa e Silva" inclui 168 organismos fitoplanctónicos, de entre os quais 128 são cianobactérias.

[2] Agolada de Baixo, Alvito, Beliche, Bravura, Caia, Corgas, Crato, Divor, Enxoé, Funcho, Magos, Maranhão, Montargil, Mte. da Barca, Mte. da Rocha, Mte. Novo, Odivelas, Patudos, Roxo, Torrão, Toulica.

O teor de microcistinas detectado em amostras naturais é muito variável (ver 3.2 e tabela 4). Nalguns reservatórios é comum manter-se um nível persistente, mas residual de microcistinas, mas têm sido também detectados com frequência níveis superiores ao valor-guia da OMS para águas de consumo (ver 3.3).

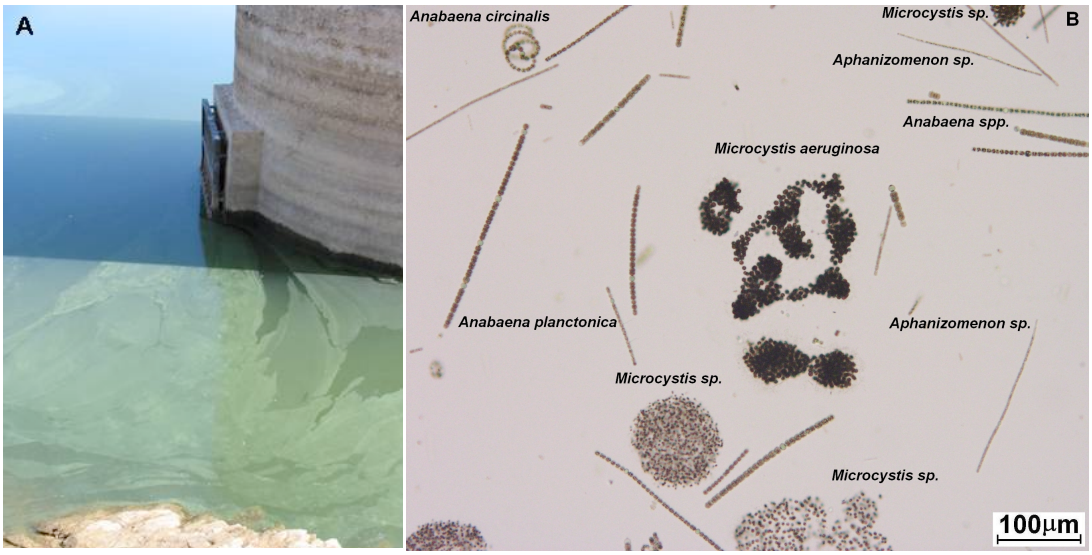


Figura 1. Aspecto macroscópico de uma florescência cianobacteriana junto à torre de captação de água na albufeira do Roxo em 2005 (A); aspecto microscópico de um *bloom* detectado na albufeira de Odivelas em 2005, dominado pelas espécies *Microcystis spp*. e *Anabaena spp.* (B). ©2005 *Sérgio Paulino.*

2. Aspectos toxicológicos das microcistinas

2.1. Relação estrutura-actividade

As microcistinas são heptapéptidos cíclicos cuja estrutura se apresenta na figura 2. São constituídas por quatro aminoácidos D conservados [D-alanina (D-Ala), ácido D-eritro-β-metilaspártico (D-MeAsp), ácido D-isoglutâmico (D-Glu) e N-metil-desidro-alanina (Mdha)], dois aminioácidos L variáveis (L-X e L-Z) e um aminoácido invulgar (até agora apenas identificado nas microcistinas e nodularinas) designado por ADDA, ácido (2*S*, 3*S*, 8*S*, 9*S*, 4*E*, 6*E*)-3-amino-9-metoxi-2,6,8-trimetil-10-decafenildienóico. A variação dos aminoácidos nas posições 2 e 4 e dos radicais R1 e R2 (como a desmetilação, por exemplo) resulta num conjunto de mais de 60 variantes. A variante que apresenta X= L-leucina, Z= L-arginina, R1=R2=CH_3, designada por microcistina LR (MCLR), é a mais tóxica e também a mais comum (Fawell et al., 1993). O peso

molecular das microcistinas varia entre 500 e 4000 dalton, tendo a maioria das variantes um valor entre 909 e 1115 dalton e a MCLR, 994 dalton (Duy et al., 2000).

O mecanismo de acção tóxica das microcistinas, designadamente a hepatotoxicidade aguda, deve-se à sua actividade de inibição das fosfatases proteicas PP1 e PP2A (Yoshizawa et al., 1990). A cadeia lateral hidrófoba do Adda e o grupo carboxilo do ácido glutâmico desempenham um papel fulcral na toxicidade das microcistinas (Nishiwaki-Matsushima et al., 1991), estabelecendo uma interacção não covalente inicial entre a toxina e as fosfatases PP1 e PP2A. Por outro lado, a N-metil-desidro-alanina (Mdha) permite estabelecer uma ligação covalente entre a microcistina e os resíduos de cisteína cys-273 e cys-266 das fosfatases PP1 e PP2A, respectivamente (MacKintosh et al., 1995; Runnegar et al., 1995), que não sendo estritamente necessária para a inibição dos enzimas, contribui para o aumento da afinidade das microcistinas (Dawson, 1998; Bischoff, 2001).

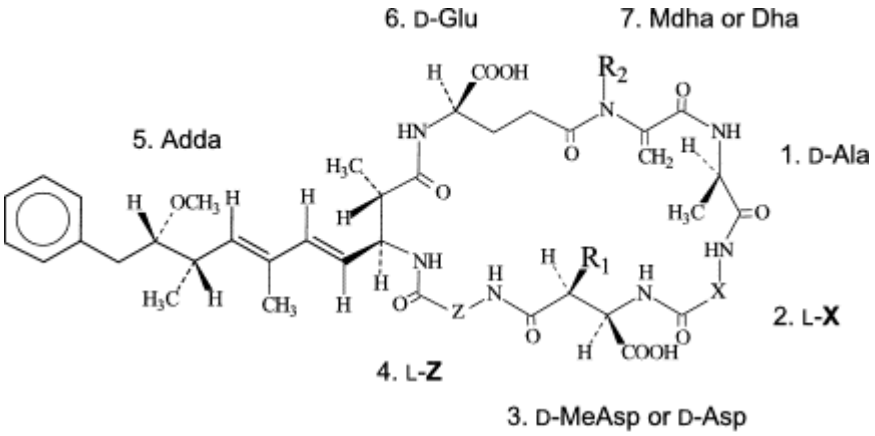


Figura 2. Estrutura química das microcistinas (Chen et al., 2006).

As microcistinas são moléculas anfipáticas contendo funções hidrófilas (grupos carboxilo e grupo guanidino da arginina) e hidrófobas (resíduo ADDA) (Vesterkvist e Meriluoto, 2003). Modificações na estrutura-base resultam em variantes com graus de hidrofilicidade/lipofilicidade e polaridade distintos, o que se repercute em diferenças na toxicocinética (Vesterkvist e Meriluoto, 2003), e em variantes com afinidades diferentes para as fosfatases proteicas (Dawson e Holmes, 1999; Chen et al., 2006). Ambos os

factores se traduzem em graus de toxicidade diferentes, tal como se exemplifica na tabela 1. As variantes LR, LA e YR são as que apresentam um grau de toxicidade mais elevado; as variantes desmetiladas em R1/R2 e a MCWR, caracterizam-se por uma toxicidade intermédia e as variantes RR, M(O)R e LY são as menos tóxicas (Harada, 1996; Moreno et al., 2003).

As microcistinas são compostos hidrossolúveis que podem persistir até cerca de 20 dias na água após tratamento com algicidas para destruição de fluorescências (Jones e Orr, 1994), sendo decompostas naturalmente através de biodegradação e fotólise (Kenefick et al., 1993; Tsuji et al., 1994). As microcistinas apresentam uma elevada estabilidade química, quer em termos de temperatura quer em termos de pH, resistindo a condições extremas. Aliás, são resistentes ao pH do estômago e não sofrem a acção das peptidases gástricas, sendo absorvidas intactas ao nível do duodeno e tendo como órgão-alvo principal, o fígado.

Tabela 1. Exemplos de variantes estruturais e toxicidade de microcistinas (adaptado de Sivonen e Jones, 1999).

Variante de microcistina	**X**	**Z**	**R1**	**R2**	**LD_{50} (µg/Kg) (i.p., murganho)**
Microcistina-LR	Leu	Arg	CH_3	CH_3	50
Microcistina-LA	Leu	Ala	CH_3	CH_3	50
Microcistina-YR	Tyr	Arg	CH_3	CH_3	70
Microcistina-RR	Arg	Arg	CH_3	CH_3	600
Desmetil 3,7 – microcistina-LR	Leu	Arg	H	H	300

2.2. Absorção, distribuição, metabolização e eliminação (ADME)

A informação disponível sobre a toxicocinética das microcistinas tem sido obtida através de estudos em animais administrados intravenosa (i.v.) ou intraperitonealmente (i.p.) (Kuiper-Goodman et al. 1999). Muito possivelmente porque os custos de aquisição de toxinas para os ensaios de administração oral são proibitivos,

atendendo ao facto da toxicidade oral das microcistinas ser 100 vezes menor que a toxicidade por administração i.p. (tabela 2).

Os primeiros estudos sobre a toxicocinética das microcistinas foram conduzidos em ratos e murganhos administrados i.v. com toxina marcada radiactivamente com ^{125}I e ^{3}H, respectivamente (citados em Stotts et al., 1997a). Estes autores verificaram que a microcistina é rapidamente removida da corrente sanguínea e concentrada no fígado, processo caracterizado por uma curva bifásica com tempos de semi-vida $t_{1/2}$ (α) = 2,1 min. e $t_{1/2}$ (β) = 42 min. nos ratos. Nos murganhos este processo é ainda mais rápido com $t_{1/2}$ (α) = 0,8 e $t_{1/2}$ (β) = 6,9 min. (Stotts et al., 1997a). Em suínos administrados i.v. com [3H]2H-MCLR foi também observada a remoção rápida e bifásica da toxina do sangue e a acumulação no fígado. Porém, nestes, a clearence sanguínea é mais lenta do que em roedores e parece depender da dose de toxina [$t_{1/2}$ (α) = 3 - 4 min. e $t_{1/2}$ (β) = 2,2 - 4,5 h] (Stotts et al., 1997a). A capacidade de eliminar a microcistina poderá variar com as espécies, o que poderá justificar as diferenças referidas (Duy et al., 2000).

A MCLR é absorvida nos intestinos, sobretudo através dos transportadores dos ácidos biliares no *ileum*, mas também por difusão passiva no *jejunum* (Dahlem et al., 1989), transportada para a corrente sanguínea e concentrada no fígado (Bischoff, 2001). O organotropismo das microcistinas resulta do facto destas toxinas entrarem nos hepatócitos através do sistema de transporte activo OATP - *Organic Anion Transporter Polypeptide* (Fisher et al., 2005). Este sistema, presente em vários órgãos dos mamíferos, constitui uma superfamília de transportadores transmembranares que permitem a passagem de um vasto espectro de solutos orgânicos anfipáticos, componentes essenciais dos processos metabólicos e fisiológicos naturais. O fígado expressa selectivamente alguns destes transportadores (OATPA, OATPB, OATPC, OATP1, OATP4, OATP8) envolvidos no transporte de muitos agentes xenobióticos cuja biotransformação e eliminação ocorre por via hepática, enter os quais as microcistinas (Hagenbuch e Meier, 2003; Fisher et al., 2005). Porém, alguns destes transportadores (OATPA, OATPB) também são expressos noutros órgãos, tais como rins, intestinos e cérebro ainda que numa menor proporção (Hagenbuch e Meier, 2003).

Estudos acerca da distribuição das microcistinas em murganhos demonstraram que entre 50 a 70% da dose total de toxina administrada por i.p. ou i.v. se acumula no fígado, 7 a 10% nos intestinos e 1 a 5% nos rins (Robinson et al.1989; Meriluoto et al., 1990). Um padrão de distribuição similar foi observado num estudo em suínos (Stotts et

al., 1997b), mas, no entanto, a acumulação da MCLR no fígado de rato é inferior (20%) (citado em Duy et al., 2000). Para além do fígado, intestinos e rins, as microcistinas também se distribuem pelo coração, baço, cérebro, gónadas e estômago, ainda que em proporções muito reduzidas, de acordo com estudos em ratos (Wang et al., 2008) e suínos (Stotts et al., 1997b). De facto, evidências crescentes têm demonstrado que para além dos efeitos hepáticos as microcistinas também exercem toxicidade nos rins (Nobre et al., 1999; Milutinović et al., 2002, 2003; Moreno et al, 2005; Andrinolo et al., 2008), intestinos (Botha et al, 2004; Gaudin et al, 2008a), pulmões (Soares et al., 2007), sistema reprodutor (Ding et al, 2006) e cérebro (Maidana et al., 2006), apesar dos processos toxicocinéticos e toxicodinâmicos das microcistinas nestes órgãos serem ainda desconhecidos.

Alguns trabalhos têm demonstrado que no fígado as microcistinas se conjugam com com o glutationo reduzido (GSH) e a cisteína (Cys), reacções da fase II dos processos de biotransformação/destoxificação. No entanto, o conhecimento acerca da metabolização das microcistinas é ainda muito limitado. Num estudo com murganhos administrados i.v. com MCLR tritiada, Robinson et al. (1990) sugeriram, pela primeira vez, a formação de eventuais produtos de destoxificação da MCLR. Num estudo *in vivo* com ratos e murganhos tratados com MCLR e MCRR, Kondo et al. (1996) observaram que 3 h e 24h após administração i.p., uma pequena percentagem das microcistinas era detectada na forma conjugada com GSH e Cys, respectivamente. Os autores concluíram que estes metabolitos são formados pela ligação dos grupos tiol do GSH (figura 3) e da Cys com o aminoácido Mdha das microcistinas. Neste estudo foi também identificado outro metabolito resultante não só da conjugação de Mdha com GSH, mas também da epoxidação, hidrólise e sulfatação do aminoácido ADDA, o que pressupõe o envolvimento de enzimas da fase I no processo de destoxificação das microcistinas. Ito et al. (2002), num ensaio com murganhos, demonstraram que os metabolitos MCLR-GSH e MCLR-CYS apresentam uma toxicidade menor que a MCLR (cerca de 12 vezes, similar à toxicidade da variante MCRR), embora com uma actividade inibidora das fosfatases PP1 e PP2A idêntica à da toxina não metabolizada. A conjugação de microcistinas com GSH foi também descrita em inúmeros organismos aquáticos, desde plantas a peixes (Pflugmacher et al., 1998). Estes trabalhos sugerem, assim, que o GSH desempenha um papel central na metabolização e destoxificação das microcistinas.

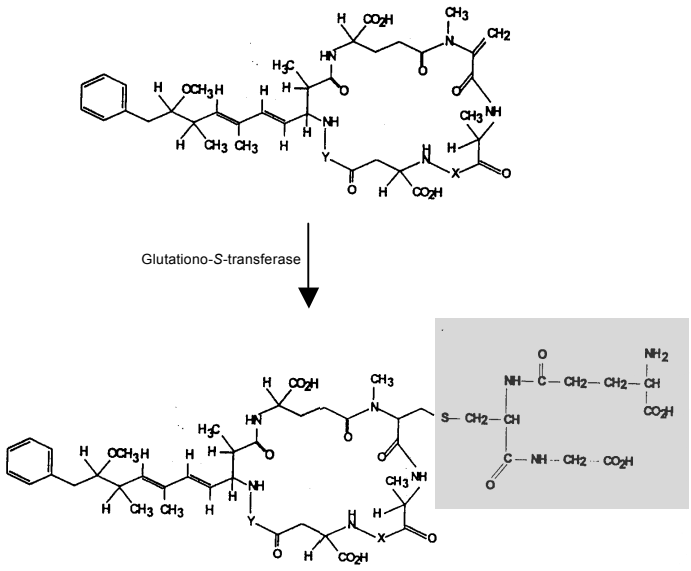


Figura 3. Conjugação da MCLR com o glutationo (adaptado de Pflugmacher e Wiegand, 2001).

Esta hipótese tem sido também suportada pela observação de que a exposição a MCLR reduz o nível de GSH (Ding e Ong, 2003) e que a conversão das microcistinas no fígado em compostos mais polares se correlaciona com a depleção de GSH (Pfugmacher et al., 1998). Porém, resultados de outros estudos com suínos, roedores e peixes (Bischoff, 2001) têm vindo a mostrar que a MCLR não é metabolizada e que a depleção dos níveis de GSH pode estar relacionada com a defesa antioxidante em resposta à produção de espécies reactivas de oxigénio (ROS) (Ding e Ong, 2003; Žegura et al., 2006). Está ainda por esclarecer se o *pool* de GSH intracelular é suficiente para, simultaneamente, combater o stress oxidativo e metabolizar as microcistinas e que condições de exposição determinam a actividade para a qual é canalizado o GSH disponível.

As diferenças da concentração máxima de microcistina detectada no sangue de suínos administrados no *loop* ileal relativamente aos animais administrados i.v, sugerem que o efeito da primeira passagem é, em parte, responsável pela eliminação de MCLR (Stotts et al, 1997a). No murganho, 1 hora após administração i.v. de uma dose subletal de [^{3}H]dMCLR, cerca de 24% da dose total de microcistina é excretada, 15% pela via biliar e 9% pela urina, sendo a toxina detectável nas fezes até cerca de 6 dias após a administração (Robinson et al., 1990). Quer a forma livre, quer a forma conjugada com GSH e CYS foram detectadas nas fezes e urina (Ito et al. 2002). Estes dados comprovam que embora o tempo de permanência e a dose de toxina a que o fígado está sujeito seja maior do que noutros orgãos, os intestinos e os rins, pelo seu papel na

absorção e/ou eliminação das microcistinas, constituem alvos potenciais da sua toxicidade. Na figura 4, esquematizam-se os processos de absorção, distribuição, metabolização e eliminação (ADME) da MCLR.

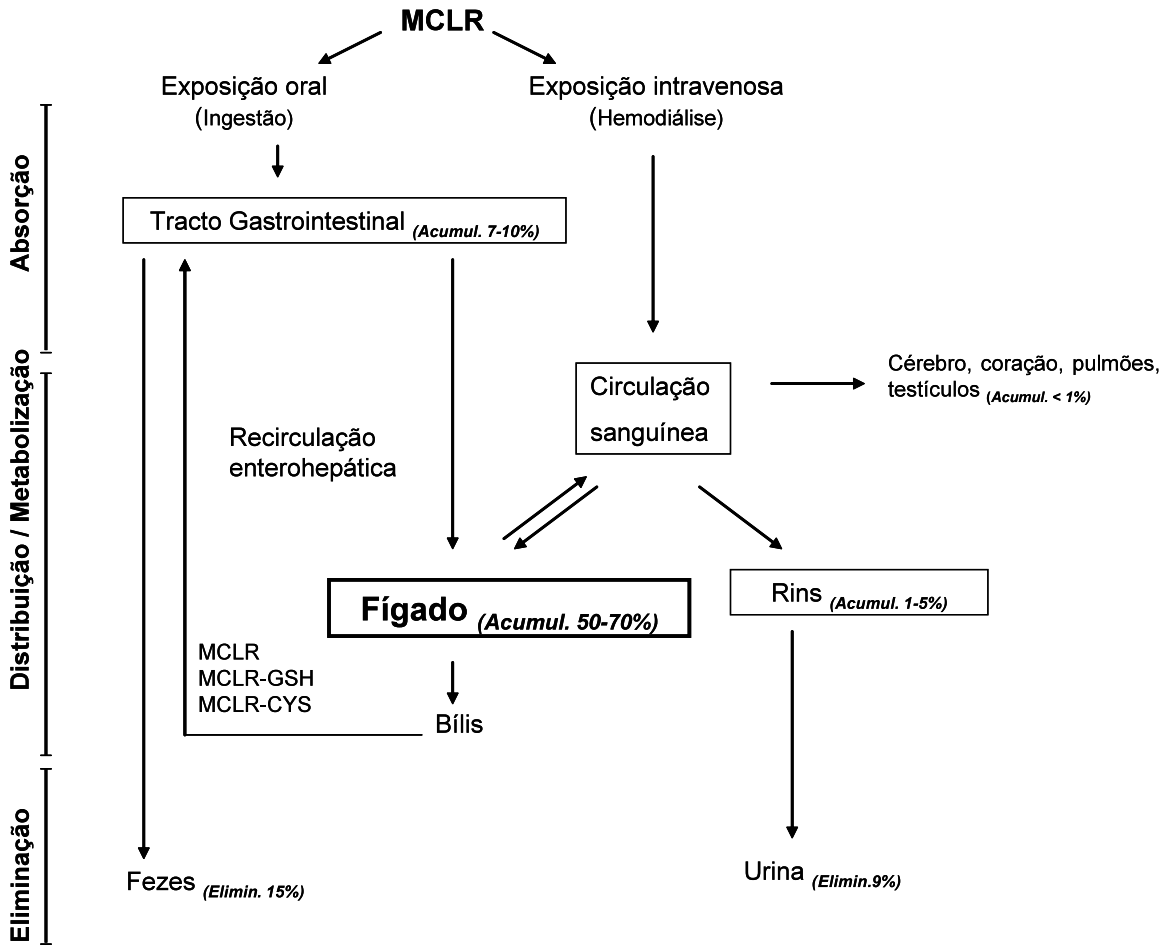


Figura 4. Representação esquemática dos processos ADME da MCLR.

A toxicidade aguda das microcistinas depende não só da variante de toxina, mas também da via de exposição (tabela 2) e da espécie de organismo exposto. Considera-se que a MCLR é cerca de 30 a 100 vezes menos tóxica pela via oral do que pela via i.p. (Fawell, 1999) devido às diferenças na toxicocinética; ou seja, na administração i.p. a toxina não sofre o processo de absorção através do trato gastrointestinal ficando directamente disponível na corrente sanguínea para ser internalizada nos hepatócitos

(Funari e Testai, 2008). Os murganhos são mais sensíveis que os ratos, pelo que constituem um modelo animal de estudo da toxicidade das microcistinas mais adequado (Rao et al., 2005). A idade parece ser outro factor que influencia a toxicidade das microcistinas uma vez que os animais mais velhos apresentam uma maior susceptibilidade à MCLR (Ito et al, 1997ª; Rao et al., 2005), muito possivelmente devido à menor capacidade de destoxificação.

Tabela 2. Toxicidade (LD_{50}) da MCLR no murganho de acordo com a via de exposição.

Via de exposição	LD_{50} (µg/Kg)	Referência
Oral	5000	Yoshida et al. (1997)
Intraperitonial	50 Valor geralmente aceite para a LD_{50} (i.p.) no murganho.	Chen et al. (2006)
	43	Gupta et al. (2003)
	65	Yoshida et al. (1997)
	65	Robinson et al. (1989)
Intratraqueal	100	Ito et al. (2001)

2.3. Mecanismos de toxicidade das microcistinas

2.3.1. Inibição das fosfatases proteicas PP1 e PP2A e alterações do citosqueleto

O mecanismo de hepatotoxicidade aguda das microcistinas é mediado pela inibição das fosfatases proteicas PP1 e PP2A (Yoshizawa et al., 1990). Estudos *in vitro* com as subunidades catalíticas das PP1 e PP2A purificadas a partir do músculo esquelético de coelho, permitiram determinar o valor de IC_{50} de 0.04 nM para a PP2A e 1.7 nM para a PP1 (Honaken et al., 1990), o que demonstra a elevada potência da MCLR como inibidora das fosfatases PP1 e PP2A.

A inibição destes enzimas perturba o equilíbrio entre os estados de fosforilação e desfosforilação, conduzindo à hiperfosforilação de proteínas envolvidas na dinâmica de organização do citoesqueleto (figura 5) que, por sua vez, induz alterações na estrutura do hepatócito (Toivola e Eriksson, 1999). Os danos estruturais nos hepatócitos têm

como consequência a perda do contacto intercelular, o colapso dos hepatócitos e das sinusóides hepáticos, a necrose e hemorragia hepática (o volume hepático aumenta notavelmente até cerca de 100% em consequência da acumulação de sangue), culminando, em situações extremas, na morte (Falconer e Yeung, 1992; Bishoff, 2001).

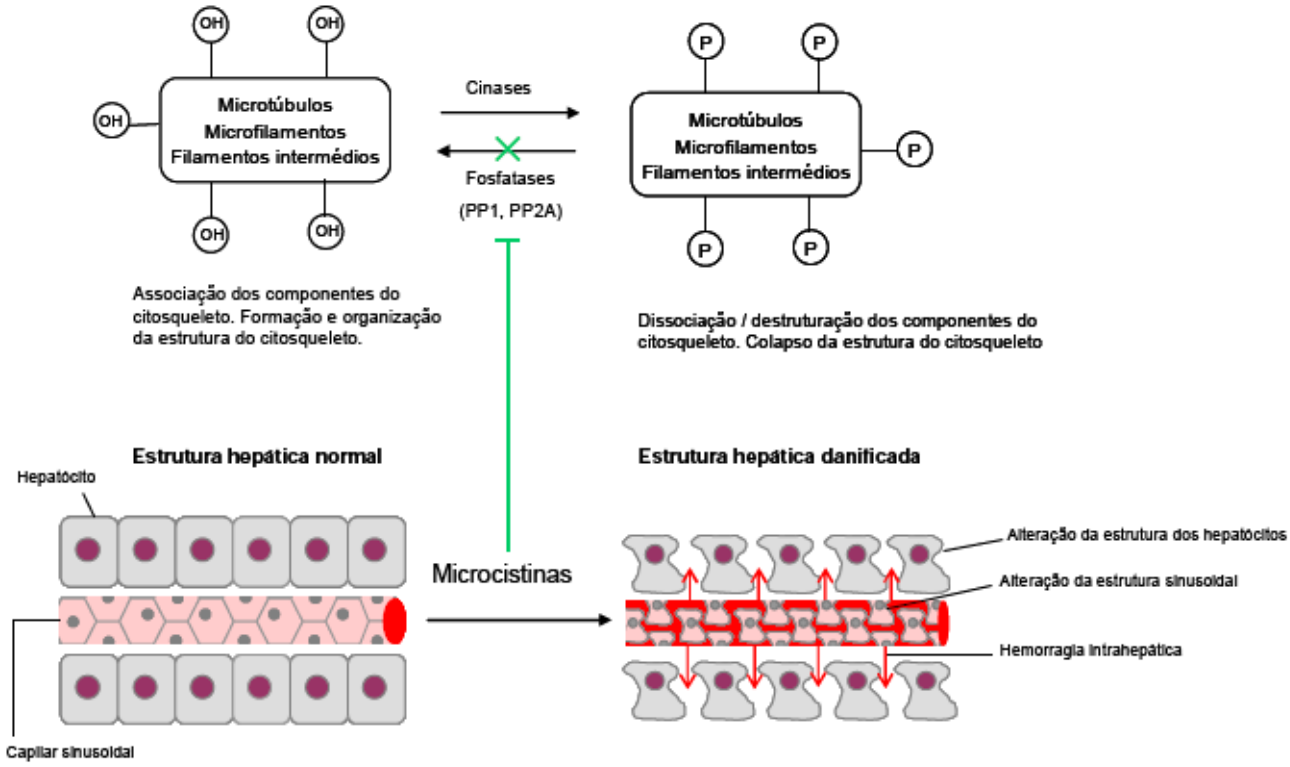


Figura 5. Esquema representativo dos efeitos das microcistinas no citosqueleto mediados pela inibição das fosfatases proteicas PP1 e PP2A (adaptado parcialmente de Boelsterli, 2009).

Os efeitos das microcistinas na morfologia e ultrastrutura do citosqueleto têm sido amplamente documentados em células hepáticas de roedores (Eriksson et al., 1990; Khan et al., 1995, 1996; Ito et al., 1997a; Toivola e Eriksson, 1999; Billam et al., 2008). Efeitos similares foram também descritos em hepatócitos humanos (Batista et al., 2003) e de peixes (Li et al., 2001; Pichardo et al., 2005), em células epiteliais de rim de rato e fibroblastos de pele (Khan et al., 1995, 1996) e embriões de coelho (Frangež et al., 2003).

O citosqueleto é constituído por três sistemas fibrilares: os microtúbulos, os microfilamentos e os filamentos intermédios (Plancha e David-Ferreira, 1999). Os microtúbulos são polímeros do heterodímero tubulina α / tubulina β e estão envolvidos

no tráfego de vesículas e organelos intracelulares, na construção do fuso mitótico e no movimento de cromossomas (Plancha e David-Ferreira, 1999). Os microfilamentos são polímeros de actina-G e estão associados, também, à movimentação intracelular e à transdução de sinais intercelulares (Plancha e David-Ferreira, 1999). Os filamentos intermédios são constituídos por vários tipos de proteínas (citoqueratinas, vimentina, lâminas) e contribuem para a estabilidade mecânica e para a organização multicelular tridimensional (Plancha e David-Ferreira, 1999).

A inibição das fosfatases PP1 e PP2A pela MCLR induz a hiperfosforilação das citoqueratinas 8, 9 e 18 e das desmoplaquinas I e II, proteínas associadas aos filamentos intermédios, e da dineína, proteína associada aos microtúbulos (Toivola e Eriksson, 1999; Gheringer, 2004). De um modo geral, a hiperfosforilação induzida pela inibição de PP1 e PP2A causa a dissociação dos filamentos intermédios, dos microfilamentos de actina e microtúbulos (Bischoff, 2001) induzindo a agregação ou colapso quer dos microtúbulos quer dos microfilamentos, o que culmina na perda da estrutura do citosqueleto (Khan et al., 1996; Ding et al., 2000a).

2.3.2. Stress oxidativo e apoptose

Para além dos efeitos das microcistinas ao nível do citosqueleto, têm também sido descritos outros efeitos a nível subcelular, em particular efeitos associados ao processo de apoptose, tais como o retraimento celular, a formação de projecções membranares (*blebs)* e a fragmentação de ADN. Estes efeitos foram descritos em hepatócitos humanos e de roedores (McDermott et al*.,* 1998; Ding et al., 2001; Mankiewicz et al., 2001; Bouaicha e Maatouk, 2004), em linfócitos humanos (Mankiewicz et al., 2001; Lankoff et al., 2004) e nalgumas linhas celulares (Rao et al., 1998; McDermott et al*.,* 1998; Lankoff et al., 2003). A capacidade das microcistinas induzirem apoptose no fígado *in vivo* foi também já confirmada em estudos com murganhos (Guzman e Solter, 1999; Hooser, 2000; Chen et al., 2005; Weng et al., 2007) e com peixes (Li et al., 2005). Alguns estudos sugerem que o stress oxidativo e as alterações na função mitocondrial são os principais processos de indução da apoptose pela MCLR. De facto, tal como foi já referido, tem sido demonstrado que a MCLR induz a produção de espécies reactivas de oxigénio (ROS) e a depleção dos níveis de glutationo reduzido (GSH) em hepatócitos de murganho (Ding e Ong, 2003). Foi também demonstrado que a MCLR induz alterações no potencial de membrana

mitocondrial (MMP) e na permeabilidade mitocondrial (MPT), com a consequente libertação de citocromo *c* e Ca^{2+} (Ding et al., 2000b, 2001), a activação de calpina e da proteína cinase II dependente do complexo Ca2+/calmodulina (Fladmark et al., 2002), alterações na cadeia transportadora de electrões e na ultraestrutura mitocondrial (Zhao et al., 2008). Outros mecanismos tais como a activação de caspases (Fladmark et al., 1999), a alteração da expressão de proteínas pró-apoptóticas da família Bcl-2 e do gene p53 (Fu et al., 2005; Weng et al., 2007; Billam et al., 2008) foram também associados à indução de apoptose pela MCLR. Alguns autores demonstraram, ainda, o envolvimento dos lisossomas no processo de apoptose induzido pela MCLR (Bouaru et al., 2006; Li et al, 2007).

2.3.3. Cancerigenicidade

O processo de cancerigénese tem sido explicado através do modelo multifaseado que, de uma forma simplificada, descreve a sequência de eventos genéticos e epigenéticos conducentes à formação de neoplasias malignas (Yuspa, 2000). Este modelo considera três fases sequenciais distintas: a iniciação, a promoção tumoral e a progressão (Yuspa, 2000). A iniciação corresponde a uma alteração irreversível no ADN (mutação), resultante de danos induzidos por agentes genotóxicos. A interacção das células iniciadas com promotores tumorais induz alterações epigenéticas (por exemplo, activação de vias de sinalização) que estimulam a proliferação das células previamente alteradas (expansão clonal). Nesta lesão pré-maligna ocorrem uma série de alterações bioquímicas e metabólicas (hipoxia, stress oxidativo, alterações de pH, interferência com os mecanismos de reparação de ADN) que criam um microambiente propício para a acumulação de danos no ADN e aumento da instabilidade genómica (por exemplo, aneuploidia) (Yuspa, 1998; Laconi, 2007). Nesta situação, surgem as condições adequadas à selecção dos clones com maior capacidade de sobrevivência, culminando na progressão para a malignidade (Laconi, 2007).

Alguns estudos epidemiológicos (ver 2.4) permitiram associar a exposição crónica humana a baixas doses de microcistinas na água de consumo e o aumento da incidência de cancro hepático (Yu, 1995; Ueno et al., 1996) e colorectal (Zhou et al, 2002). Estudos de cancerigenicidade em roedores demonstraram, também, que a MCLR promove a tumorigénese no fígado (Nishiwaki-Matsushima et al. 1992), na pele (Falconer, 1991) e no cólon (Humpage et al., 2000a) em animais previamente tratados com agentes mutagénicos. Por outro lado, Ito et al. (1997b) demonstraram que a MCLR

induz, *per si*, a formação de nódulos neoplásicos no fígado de ratinho sem uma exposição prévia a um agente iniciador tumoral, o que sugere que a MCLR possa constituir um carcinogéneo completo.

Embora a MCLR esteja classificada pela Agência Internacional para a Investigação do Cancro (International Agency for Research on Cancer, IARC, 2006) como potencialmente cancerigénica para o Homem (pertencente ao grupo 2B), os mecanismos responsáveis por essa acção estão ainda por esclarecer.

2.3.3.1. Genotoxicidade

Estudos recentes de genotoxicidade *in vivo* e *in vitro* têm revelado que a MCLR apresenta, de facto, propriedades genotóxicas, corroborando a hipótese da MCLR ser capaz de induzir alterações genéticas que poderão estar na génese do processo cancerigénico. No entanto, esta é uma matéria que tem gerado alguma controvérsia.

Os primeiros trabalhos acerca da eventual genotoxicidade de microcistinas demonstraram que extractos de estirpes de *Microcystis* sp. produtoras de MCLR não induzem mutações pontuais pelo teste de Ames (Grabow et al., 1982; Repavich et al., 1990). Mais recentemente, Wu et al. (2006) também não encontraram actividade mutagénica em extractos cianobacterianos usando os testes de mutagénese *in vitro* ara, Ames e SOS/umu. Contrariamente, Ding et al. (1999) obtiveram uma forte resposta mutagénica no teste de Ames quando testaram um extracto de *M. aeruginosa*, mas o resultado foi negativo quando testaram MCLR pura, tal como havia sido descrito previamente por Tsuji et al. (1995). Contudo, Susuki *et al.* (1998) observaram um aumento na frequência de mutação no *locus* de resistencia à oubaína em células Rsa expostas a MCLR e Zhan et al. (2004) um aumento na frequência de mutação no gene *hprt* da linha celular linfoblastóide TK6, indicando uma actividade mutagénica em genes endógenos de células somáticas humanas. Por outro lado, foi demonstrado que a MCLR não forma aductos com o ADN em células de fígado de rato (Bouaïcha et al., 2005), o que constituiria um indicador de lesão pré-mutagénica, sugerindo que a actividade genotóxica da MCLR será provavelmente mediada por um mecanismo indirecto.

Tem sido descrito nalgumas publicações que a MCLR induz quebras no ADN de células do fígado *in vivo* (Rao e Bhattacharya, 1996; Rao et al., 1998; Gaudin et al, 2008a), em hepatócitos em cultura (Ding et al., 1999; Žegura et al, 2003, 2004, 2006; Nong et al., 2007;) e noutros tipos celulares (Rao et al., 1998; Mankiewicz et al., 2002;

Lankoff et al., 2004; Žegura et al, 2008a). Porém, o mecanismo subjacente a estas lesões no ADN (avaliadas pelo ensaio do Cometa) não foi ainda claramente definido e parece ser fortemente dependente da dose e do tipo celular. De facto, alguns autores atribuíram as quebras de ADN (quantificadas no ensaio do Cometa) à degradação endonucleolítica decorrente da apoptose (Lankoff et al., 2004) ou necrose (Rao et al., 1998) e não a um efeito genotóxico. Contrariamente a esta hipótese, alguns autores mostraram que as lesões de ADN induzidas por doses sub-citotóxicas de MCLR nas linhas celulares derivadas de hepatoma humano, HepG2 (Žegura *et al*. 2003, 2004, 2006; Nong et al., 2007) e de carcinoma do cólon CaCo-2 (Žegura *et al* 2008a) eram, provavelmente, uma consequência de stress oxidativo induzido pela MCLR e, portanto, decorrentes de um efeito genotóxico indirecto. Corroborando esta hipótese, foi demonstrado em células hepáticas que doses sub-citotóxicas de MCLR induzem a formação de 8-oxo-desoxiguanina, um marcador de dano oxidativo no ADN (Maatouk et al., 2004; Bouaïcha et al., 2005).

A inibição da reparação de danos no ADN é outro mecanismo indirecto de genotoxicidade que poderá contribuir para os efeitos genotóxicos da MCLR. Lankoff et al. (2006a,b) observaram que a MCLR inibe a reparação de danos induzidos pela radiação UV e gama em células CHO-K1 e em linfócitos humanos, respectivamente. Gaudin et al. (2008b) colocaram também esta hipótese na tentativa de explicar os resultados negativos do teste UDS (Unscheduled DNA Synthesis) em ratos administrado i.v. com MCLR. Este teste indica se um composto induz danos no ADN ao quantificar a síntese de ADN *de novo* na sequência de um processo de reparação por excisão nucleotídica.

A pesquisa de efeitos das microcistinas ao nível cromossómico está descrita num número muito reduzido de publicações. Lankoff et al. (2004, 2006b) não detectaram a presença de aberrações cromossómicas em linfócitos humanos expostos a MCLR. De acordo com esses resultados, Fessard et al. (2004) e Lankoff et al. (2006a) não observaram indução de MN na linha celular CHO-K1 após tratamento com MCLR. Porém, noutros trabalhos, foi já descrito um aumento da frequência de micronúcleos (MN) em eritrócitos de ratinho (Ding et al., 1999) e na linha linfoblastóide humana TK6 (Zhan et al., 2004). Os MN são corpúsculos intacitoplasmáticos constituídos por fragmentos cromossómicos ou cromossomas inteiros que não são incorporados nos núcleos das células-filhas durante a divisão celular (Fenech, 2000). A formação de micronúcleos reflecte, assim, um efeito genotóxico decorrente de um mecanismo de

acção clastogénico ou aneugénico, respectivamente. Até ao presente ainda não foi descrito o mecanismo de formação dos micronúcleos induzidos pela MCLR. A elucidação desse mecanismo será bastante importante, não só para a investigação da cancerigénese da MCLR, mas também do ponto de vista da avaliação e da quantificação do risco de exposição a microcistinas, uma vez que a distinção entre agente aneugénico e clastogénico determinará ou não, respectivamente, a possibilidade de estabelecer um limiar de exposição (Kirsch-Volders et al., 2002; Bolt et al., 2004; Iarmarcovai et al., 2006). Tem sido defendido que para agentes aneugénicos, cujo efeito é indirecto e envolve mais do que um alvo, é possível determinar a dose abaixo da qual não há efeito adverso (NOAEL, *No Observed Adverse Effect Level*), enquanto que para agentes clastogénicos que actuem directamente no ADN, qualquer exposição causará efeito adverso (Kirsch-Volders et al., 2002; Bolt et al., 2004).

Na figura 6 sumarizam-se os efeitos genotóxicos (directos e indirectos) da MCLR até agora sugeridos.

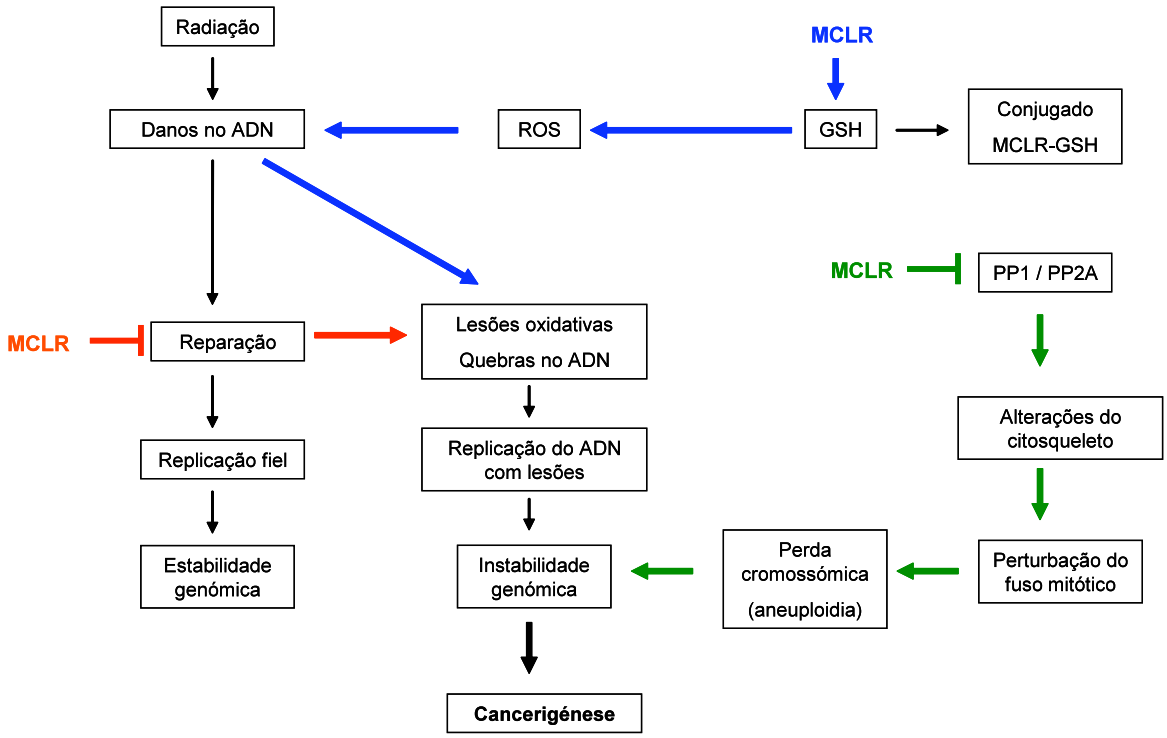


Figura 6. Esquema representativo de eventuais mecanismos de genotoxicidade induzidos pela MCLR.

2.3.3.2. Promoção tumoral

Apesar da MCLR ser considerada um potente promotor tumoral, na medida em que é capaz de induzir a transformação em células ou organismos previamente expostos a um agente genotóxico, desconhecem-se ainda os mecanismos através dos quais esse efeito é exercido. No entanto, tem sido sugerido que a actividade de promoção tumoral da MCLR é mediada pela inibição das fosfatases proteícas PP1 e PP2A, uma vez que estes enzimas desempenham um papel crucial na regulação de inúmeros processos celulares tais como a divisão e a proliferação celular, designadamente através da activação das cinases proteicas activadas por mitogéneos (MAPK - Mitogen-activated protein kinases) (Gehringer, 2004).

As MAPK são enzimas eucarióticos conservados, envolvidos nas vias de sinalização que regulam, através de cascatas de fosforilação, quase todos os processos celulares tais como a expressão génica, a proliferação celular, a motilidade e a morte celular. As MAPK dos mamíferos dividem-se em quatro grupos distintos: ERK1/2 (extracellular signal-related kinases), JNK (Jun amino-terminal kinases), proteínas 38 e ERK5, que, de um modo geral, são activados por cinases distintas e regulam funções celulares diversas (Chang e Karin, 2001).

As cinases ERK1/2 são expressas de forma ubíqua nas células dos mamíferos, fosforilam uma vasta gama de substratos em todos os compartimentos celulares e desempenham um papel central no controlo da proliferação celular através de três processos principais: 1) estimulação da síntese de ADN através da fosforilação de carbamoil fosfato sintetase II (enzima envolvido na biosíntese de pirimidinas); 2) estimulação da progressão celular através da inactivação de MYT1 (cinase inibidora do ciclo celular); 3) estimulação da actividade do complexo AP-1, com a consequente indução da ciclina D1 (Chang e Karin, 2001; Meloche e Pouysségur, 2007).

A via de sinalização ERK1/2 é activada sobretudo por factores de crescimento e agentes mitogénicos (figura 7). Ao ligarem-se ao receptor de membrana RTK (receptor tyrosine kinase), activam-no através de dimerização, autofosforilação e ligação a enzimas e proteínas adaptadoras como a Shc o que, por sua vez, activa a GTPase Ras. Esta proteína-G de membrana passa de um estado inactivo (ligada a GDP) a uma forma activa (ligada a GTP) e inicia o processo de activação, em cascata, da via de sinalização ERK1/2 (McKay e Morrison, 2007). Na forma activa, a proteína Ras recruta a cinase citoplasmática Raf tornando-a activa. Subsequentemente, a Raf fosforila as cinases MEK (isoformas 1 e 2) em dois resíduos de serina que, por sua vez, têm como substrato

as cinases ERK (isoformas 1 e 2), fosforilando-as num resíduo de tirosina e treonina. Para que a transmissão do sinal entre os vários componentes da cascata Ras-Raf-MEK-ERK seja eficaz, é necessário maximizar a sua proximidade, através da ancoragem dos efectores a montante e a jusante da via em proteínas *scaffold* tais como KSR (cinase supressor of Ras) e MP1 (MEK binding protein) (Kolch, 2000; Junttila et al., 2008).

As formas activas de ERK1/2 são translocadas para o núcleo, induzindo a activação de vários factores de transcrição tais como c-Fos e c-Jun (complexo transcripcional AP1), c-Myc e Ets1 (Junttila et al., 2008), desencadeando, assim, a proliferação celular.

O balanço entre a fosforilação (catalisada por cinases) e a desfosforilação (catalisada por fosfatases) tem um papel fundamental na regulação e propagação do sinal na cascata Ras-Raf-MEK-ERK. Todos os passos da via podem ser regulados por fosfatases (Junttila et al., 2008). De acordo com a especificidade para os substratos, foram caracterizados três tipos de fosfatases que actuam na via de ERK1/2: MKPs (*MAPK phosphatases*), PSPs (*protein serine/threonine phosphatases*) e PTPs (*protein tyrosine phosphatases*). As fosfatases proteicas PP1 e PP2A incluem-se no grupo das PSPs, que removem especificamente grupos fosfato em resíduos fosforilados de serina e treonina. A PP2A, em particular, desempenha sobretudo uma função de inibição da via ERK1/2: liga-se à proteína Shc ao nível do receptor de membrana, inibindo a activação da Ras e, portanto, a propagação do sinal, e inactiva as cinases MEK e ERK (Junttila et al., 2008). No entanto, também pode activar a proteína KSR e a cinase Raf, através da desfosforilação de locais de inibição, promovendo a sua activação pela Ras (Raman et al., 2007).

Para além do seu papel na indução da proliferação, as cinases ERK1/2 também medeiam processos conducentes à sobrevivência celular. A forma activa de ERK1/2 induz a fosforilação da cinase RSK que inactiva, por fosforilação, a proteína apoptótica BAD. Por outro lado, a RSK activa o factor de transcrição CREB que regula positivamente a transcrição das proteínas anti-apoptóticas Bcl-2, Bcl-xl e Bcl-1 (Junttila et al., 2008).

A estimulação do crescimento celular e a inibição da apoptose podem, assim, constituir os mecanismos subjacentes ao efeito de promoção tumoral das microcistinas (Gheringer, 2004).

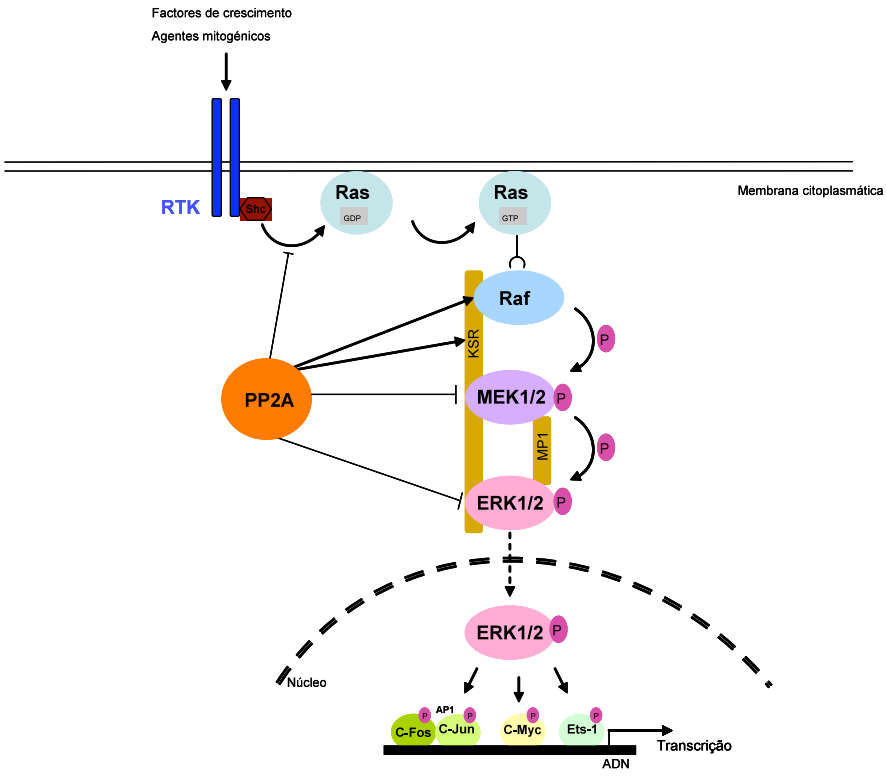


Figura 7. O papel da fosfatase proteíca PP2A na regulação da via de sinalização Ras-Raf-MEK1/2-ERK1/2.

Legenda: **AP1** (*activator protein-1*); **c-Fos, c-Jun, c-Myc, ETS-1** (factores de transcrição); **ERK** (*extracellular-signal-regulated kinase*); **GDP** (difosfato de guanosina); **GTP** (trifosfato de guanosina); **KSR** (*kinase supressor of Ras*); **MAP** (*mitogen activated protein*); **MEK** (*MAP kinase kinase*)**; MP1** (*MEK partner 1*); **PP2A** (fosfatase proteica do tipo 2A); **P** (grupo fosfato); **Raf** (*MAP kinase kinase kinase*); **Ras** (GTPase); **RTK** (*receptor tyrosine kinase*); **Shc** (proteína adaptadora); ↓ activação, ⊥ inibição.

O envolvimento da via ERK1/2 na promoção tumoral induzida pelas microcistinas tem sido suportado por alguns estudos. Li et al. (2009) demonstraram que um extracto de microcistinas purificado a partir de um *bloom* cianobacteriano induz a activação dos proto-oncogenes *c-jun*, *c-fos* e *c-myc* (componentes que constituem o complexo transcripcional AP-1) no fígado, rim e gónadas de ratos administrados por i.v. Zhu et al. (2005) verificaram que a MCLR tem a capacidade de transformar células da cripta do cólon imortalizadas, tornando-as independentes da adesão e estimulando a sua proliferação, através da activação das vias AKT e MAPK (p38 e JNK). Estes resultados suportam a hipótese de que a MCLR constitui um risco de cancro colorectal (Zhou et al., 2002). No mesmo estudo, Zhu et al. (2005) também verificaram que as proteínas Ras e Raf são activadas pela MCLR, sem que isso se traduza, no entanto, na activação

de ERK1/2. Contrariamente, Komatsu et al. (2007) observaram que a MCLR induz a fosforilação de ERK1/2 na linha celular renal HEK293. No entanto, este processo ocorreu para uma concentração de toxina (50 nM) que induziu apoptose, pelo que os autores concluíram que naquela linha celular o efeito apoptótico da MCLR é mediado pelas cinases ERK1/2.

2.4. Impacto das microcistinas na saúde humana

Têm sido registados inúmeros casos de mortalidade animal por ingestão de água contaminada por cianobactérias produtoras de microcistinas, sobretudo com a espécie *Microcystis aeruginosa.* Esses episódios estão descritos em países tão diversos como a Argentina, Austrália, Escócia, Finlândia, Inglaterra e Noruega, e os animais afectados têm sido sobretudo gado ovino e bovino, cães e peixes (Duy et al., 2000).

Alguns casos de intoxicação aguda humana, incluindo casos letais, por exposição a água contaminada com microcistinas têm também sido registados em alguns países (tabela 3).

O caso mais emblemático remonta a 1996 e sucedeu numa unidade de hemodiálise em Caruaru, no Brasil (revisto em Pouria et al., 1998). O episódio ocorreu num período de seca que deixou a localidade sem acesso a água tratada, pelo que a unidade de hemodiálise teve de ser abastecida com água proveniente de um reservatório superficial de uma localidade vizinha, onde tinha ocorrido um *bloom* de *Microcystis sp*. e *Anabaena sp.* Todos os 126 hemodializados desenvolveram sintomas, poucas horas após o tratamento, tais como fraqueza, mialgia, sintomas neurológicos (dores de cabeça, vertigens, surdez, cegueira e convulsões), náuseas e vómitos, hepatomegália, alterações dos marcadores bioquímicos hepáticos, apoptose e necrose hepática e falência hepática. Cerca de 85% dos doentes apresentavam danos hepáticos evidentes, a maioria homens na faixa etária entre os 50 e 59 anos, e 60 pacientes acabaram por falecer, a maioria entre a primeira e a quinta semana após o tratamento. A análise de amostras biológicas dos pacientes revelou a presença de microcistinas (LR, YR e LA) no soro (1-10 $ng.mL^{-1}$) e no fígado (0,1-0,5 $ng.mg^{-1}$). Com base no teor de microcistinas determinado no fígado dos pacientes e no volume de exposição, foi estimada em 19,5 $\mu g.L^{-1}$ a concentração de microcistinas a que os doentes estiveram expostos (Apeldoom et al., 2007).

Para além dos efeitos agudos, alguns estudos epidemiológicos evidenciam uma possível associação entre o aumento da incidência de hepatocarcinoma (Yu, 1995; Ueno et al., 1996) e de cancro colorectal no Homem (Zhou et al, 2002) e a ingestão de água indevidamente tratada e frequentemente contaminada com *M. aeruginosa* e microcistinas.

Tabela 3. Exemplos de episódios de intoxicação humana por microcistinas (adaptado de Chorus et al., 2000; Duy et al., 2000).

País / Ano	Nº pessoas afectadas	Efeitos	Via de exposição
EUA/1931	5000-8000	Gastroenterite	Ingestão de água tratada proveniente dos rios Ohio e Potomac após a ocorrência de *blooms* intensos de *Microcystis spp.* O tratamento da água (precipitação, filtração e cloragem) não foi suficiente para remover as toxinas
Canadá/1959	13	Gastroenterite	Ingestão acidental de água de um lago durante actividades balneares. Nas fezes de um paciente foram detectadas células de *Microcystis spp*. e *Anabaena sp*.
Austrália/ 1981	(não especificado)	Aumento da actividade dos enzimas hepáticos	Ingestão de água tratada proveniente de um reservatório contaminado com *Microcystis spp.*
Brasil/1988	2000	Gastroenterite 88 óbitos	Ingestão de água fervida proveniente de um reservatório com densidade elevada de cianobactérias
Brasil/1996	126	60 óbitos	Tratamento por hemodiálise numa unidade abastecida por um reservatório com *bloom* de *Microcystis sp*. e *Anabaena sp*.
Reino Unido/1989	10	Gastroenterite Pneumonia	Ingestão acidental de água de um lago com um *bloom* de *Microcystis spp.* durante treinos militares
Suécia/1994	121 (40% da população de uma aldeia)	Náuseas, vómitos, diarreia, espasmos musculares.	Ingestão de água da rede acidentalmente misturada com água bruta contaminada com *Planktothrix agardhii*

O carcinoma hepatocelular (HCC) é uma das formas de cancro com maior incidência e mortalidade, tendo causado 607.000 óbitos a nível mundial (correspondendo a 1.1 % da totalidade das causas de mortalidade) em 2001 (Hernández et al., 2009). Na República Popular da China, o HCC é a segunda causa de morte por cancro, correspondendo a uma taxa de mortalidade de 20/100.000 em 1990 e tendo causado 28.463 mortes em 1999 (Lian et al., 2006). Os principais factores de risco de HCC são a infecção crónica com os vírus da hepatite B e C e a ingestão de alimentos

contaminados com aflatoxinas. Porém, o consumo continuado de água contaminada com microcistinas é actualmente considerado como um factor de risco emergente (Lian et al., 2006; Hernández et al., 2009). Observou-se, com efeito, que a co-ingestão de MCLR com aflatoxina B1 aumenta para o triplo a incidência do HCC induzido por aflatoxinas em animais experimentais (Lian et al., 2006). O cancro colorectal tem vindo a aumentar nos países "desenvolvidos" e está associado ao consumo de proteína e gordura animal, bem como ao consumo de bivalves contaminados com ácido ocadáico (uma ficotoxina produzida por fitoplâncton marinho que, tal como as microcistinas, é um inibidor potente das fosfatases proteicas) (Hernández et al., 2009). O estudo epidemiológico de Zhou et al. (2002) demonstrou que a ingestão de água contendo 50 $pg.mL^{-1}$ de microcistinas aumenta em cerca de 8 vezes o risco de cancro colorectal.

Muito possivelmente, a frequência de intoxicação humana e a extensão das populações afectadas é maior do que aquela se pode inferir pelos dados disponíveis. Isto deve-se, em grande medida, ao facto da sintomatologia associada à intoxicação com microcistinas não ser específica destes compostos, contrariamente à de outras ficotoxinas, como por exemplo a saxitoxina que causa uma dormência típica dos lábios e dedos (Kuiper-Goodman et al., 1999). A exposição humana a microcistinas induz um conjunto de sinais e sintomas facilmente atribuíveis a patologias como hepatite, cirrose, cancro hepático e alcoolismo. Não existem, portanto, biomarcadores de efeito que permitam estabelecer uma relação causal entre a exposição a microcistinas e um quadro clínico específico de intoxicação humana. Por outro lado, o desconhecimento ainda profundo acerca dos processos de biotransformação e eliminação das microcistinas não permitiu, ainda, definir um biomarcador de exposição específico que permita identificar ou prever o risco de intoxicação humana. Um dos problemas associados aos estudos toxicocinéticos com microcistinas tem sido a dificuldade de detectar quantidades vestigiais de microcistinas ou derivados, normalmente por HPLC e MS, em amostras de tecidos ou fluidos que, naturalmente, apresentam uma proporção elevada de compostos interferentes. A utilização de métodos de purificação por imunoafinidade permitiram, de alguma forma, aperfeiçoar o processo de purificação e clarificação das amostras, e ultrapassar, em parte, essa dificuldade (Kondo et al., 1996). No entanto, a incapacidade analítica para detecção de níveis vestigiais é certamente ainda uma limitação à monitorização de populações (WHO, 2003).

A via de exposição humana a microcistinas também dificulta a identificação e quantificação do risco. De facto, essa exposição ocorre sobretudo pela ingestão de água

contaminada ou pela contaminação sanguínea em unidades de hemodiálise. Contudo, alguns trabalhos revelam a bioacumulação de microcistinas em bivalves, lagostins de água doce, peixes e plantas irrigadas com água contaminada (Vasconcelos 2001a,b; Codd et al., 2005; Wiegand e Pflugmacher, 2005; Ibelings e Chorus, 2007). Nalguns países é também comum o consumo de suplementos alimentares à base de *pellets* cianobacterianos (Dietrich e Hoeger, 2005; Funari e Testai, 2008). Assim, os alimentos podem constituir uma fonte adicional de toxinas relativamente à exposição humana por via oral.

Dado os condicionalismos que impossibilitam a detecção atempada e, portanto, a prevenção, dos riscos de exposição humana a microcistinas, é fundamental a sensibilização das autoridades de saúde e do ambiente para o impacto destas toxinas e para a importância de estabelecer programas de monitorização adequados. Isto aplica-se, sobretudo, a regiões onde a água para abastecimento público é proveniente de reservatórios de água superficial, ou onde se desenvolve actividade balnear em rios, barragens e albufeiras frequentemente contaminados com cianotoxinas. Contudo, a implementação de medidas correctivas e preventivas do risco para as populações, mesmo em reservatórios frequentemente monitorizados, poderá nem sempre ser eficaz uma vez que a ocorrência de efeitos nas populações poderá estar desfasada no tempo relativamente à ocorrência de *blooms* tóxicos. Por um lado, após o decaimento do *bloom*, desaparece o sinal visual de risco, embora as toxinas possam permanecer por mais tempo na água. Por outro, o teor de cianobactérias e microcistinas pode ser muito residual mas persistente, o que poderá acarretar um risco, dificilmente identificável, de exposição crónica a baixos níveis de toxinas. Saliente-se, ainda, que algumas espécies de cianobactérias, tais como *Planktothrix rubescens* (Paulino et al., 2009), não formam *blooms* superficiais, pelo que estes poderão ocorrer a níveis mais profundos nas massas de água, eventualmente coincidindo com o ponto de captação de água para a estação de tratamento. Neste caso, a simples inspecção ao local de captação não será suficiente para detectar a presença de cianobactérias. O cuidado com a amostragem, nomeadamente a frequência das colheitas e o tipo de amostragem (superficial ou composta – combinação de amostras colhidas a várias profundidades), são aspectos fundamentais a considerar no estabelecimento de programas de monitorização de cianobactérias e toxinas associadas.

3. Monitorização ambiental de microcistinas

3.1. Métodos de detecção de microcistinas

Um dos métodos mais utilizados na quantificação de microcistinas é o método imunoquímico ELISA ("Enzime Linked Immunosorbent Assay"). Para o efeito, existem comercialmente disponíveis kits que possibilitam a análise relativamente rápida de um número elevado de amostras. Por este motivo, é uma metodologia bastante útil na monitorização de cianobactérias e toxinas associadas em reservatórios de água doce. O método baseia-se na competição entre a toxina das amostras a analisar e um análogo estrutural das microcistina imobilizado na superfície de placas de 96 poços, pela ligação a anticorpos que reconhecem o aminoácido ADDA em solução. Após a remoção da fração ligada (por lavagem) é adicionado um anticorpo secundário marcado com o enzima HRP (Horse Radish Peroxidase) cujo producto de reacção com o substrato respectivo apresenta um sinal espectrofotométrico inversamente proporcional à quantidade de microcistina presente na amostra a analisar. O limite de quantificação deste método é de 0.1 $\mu g.L^{-1}$ de microcistinas. Este método apresenta como principal desvantagem a impossibilidade de distinguir as várias variantes de microcistinas, uma vez que apenas permite quantificar o teor total de microcistinas.

A cromatografia líquida de alta pressão (HPLC - High Performance Liquid Chromatography) com detecção por UV ou por díodos (DAD) com base no princípio de partição em colunas de sílica de fase reversa é o método *standard* (ISO 20179) para a identificação e quantificação de microcistinas. O teor de microcistinas na amostra é confirmado e quantificado pela comparação dos tempos de retenção, área dos picos dos cromatogramas e espectro de absorção das amostras com os de soluções padrão. O limite de quantificação deste método é de 1 $\mu g.mL^{-1}$. Embora permita identificar entre várias variantes de microcistinas, essa identificação está restringida apenas àquelas para as quais existe um padrão comercialmente disponível.

As maiores limitações metodológicas relativamente à detecção de microcistinas são a ausência de um método robusto e simultaneamente específico e inexistência de padrões certificados para todas ou, pelo menos, para um número significativo de variantes de microcistinas.

3.2. Níveis ambientais e de exposição humana

A consulta bibliográfica sobre a ocorrência de microcistinas nos reservatórios de água doce revela que o teor de microcistinas na água bruta é bastante variável. Tal como se referiu no ponto 1.2., a produção de cianotoxinas depende de uma série de factores tais como a densidade e composição dos *blooms* e as condições ambientais. A concentração de microcistinas em amostras ambientais é frequentemente determinada na biomassa (correspondendo à fracção intracelular e expressa em $\mu g.g^{-1}$ de peso seco) ou na água bruta, quer em termos da fracção de toxinas dissolvidas (extracelular), quer em termos da fracção total (intracelular + extracelular), valores expressos em $\mu g.L^{-1}$. Na tabela 4 apresentam-se alguns exemplos dos níveis de microcistinas detectados em água bruta no âmbito da monitorização de reservatórios superficiais (rios, albufeiras, lagoas), usados para produção de água de consumo e/ou para actividades recreativas. Indica-se, também, o teor de microcistinas detectados em água tratada.

Embora não se possa definir um padrão sazonal e geográfico relativamente à ocorrência de cianobactérias tóxicas, a sua proporção em determinadas regiões durante os meses mais quentes pode ser elevada, o que se poderá traduzir em níveis de microcistinas preocupantes. Como se poder constatar pela tabela 4, o teor de microcistinas na água bruta poderá atingir níveis susceptíveis de causar intoxicação no Homem.

Relativamente a água bruta, foi efectuado um cálculo aproximado do volume de água que seria necessário ingerir para induzir um caso de intoxicação humana letal (Chorus e Fastner, 2001). Foi traçado o pior cenário com base na facto das crianças serem os indivíduos mais sensíveis porque ingerem um volume de água maior do que os adultos relativamente ao seu peso corporal (Kuiper-Goodman et al., 1999). Assim, considerando que uma criança com o peso de 10 Kg ingere água da margem de uma albufeira onde se acumula uma florescência cianobacteriana e que a toxicidade oral aguda da MCLR para o Homem é equivalente à do murganho (5-10,9 $mg.Kg^{-1}$), 50 mg de toxina poderá corresponder a uma dose letal. De acordo com o valor máximo de toxina detectado em água bruta (25.000 $\mu g.L^{-1}$), o volume de água necessário para causar a morte seria de 2L. A ingestão deste volume de água poderá ser pouco provável, mas a ingestão de um volume mais baixo poderá eventualmente causar efeitos tóxicos menos graves. Porém, e considerando que o teor de MCLR na célula cianobacteriana é de 3 $\mu g.mm^{-3}$ de biovolume, o que equivale a 3 $mg.mL^{-1}$ de material celular, a ingestão

de 17 mL de biomassa cianobacteriana poderá ser letal para uma criança (Chorus e Fastner, 2001). Este exemplo corresponderá apenas a um caso extremo de ingestão de material celular, mas revela bem o risco de efeitos agudos graves que poderão decorrer da exposição humana a água contaminada com um *bloom* cianobacteriano intenso e produtor de MCLR.

Tabela 4. Teor de microcistinas detectado em amostras de água bruta e tratada.

Local	Data	Teor de microcistinas (µg MCLR eq.L^{-1})			Referências
		Água bruta		Água tratada	
		Fracção extracelular	Total		
Alemanha	1995-97	0,1 - 16	-	-	Fastner et al., 2001
	1993-94	0,07 - 0,76	0,15 - 36	-	Citado em Sivonen e Jones, 1999
	1997	-	1 - 25.000	-	
	1996-97	≤1,0	1,0 - 10	0,07 - 0,11	Kruschwitz et al, 2001
	1998	-	7,5 - 10	0,1 - 0,3	Chorus et al., 2001
Austrália	1993	1300 - 1.800	-	-	Jones e Orr, 1994
Canadá	1990-92	0,15 - 4,3	-	0,09 – 0,64	Gupta et al., 2001
	1995	0,1 - 1,0		0,1 – 0,6	
		0,15 - 2,28		0,05 – 0,18	
China	1993-94	0.05 - 1,6	-	-	Citado em Sivonen e Jones, 1999
	-	0 - 55	-	-	Citado em Duy et al., 2000
Coreia	1992-96	-	0,4 - 171	-	Park, 2001
EUA	1993	0,07 - 200	-	-	Citado em Sivonen e Jones, 1999
Finlandia	1993-94	0,001 - 0,21	-	-	Citado em Fastner et al., 2001
Japão	1992-95	0,02 - 3,8	0,04 - 480	-	Citado em Sivonen e Jones, 1999
	1993-95	0 - 5,6	0,05 - 1.300		
	1993-94	0,08 - 0,8	0,06 - 94		
	1989-94	-	300 - 19,500		
Portugal	1994-98	-	0,2 - 31,0	-	Vasconcelos, 2001b
	2005	0,8 – 1,1	-	0,0	Paulino et al., 2009
	2005-06	-	0 – 7,2	-	Valério et al., 2008
	2005-06	-	0,5 – 222	-	Valério et al., 2009b
	1999-05	-	≤1 – 6,8	-	Galvão et al, 2008
Reino Unido	1992	-	17 - 131	-	Citado em Sivonen e Jones, 1999
República Checa	1999	0,09 – 8,7	-	0,89 – 7,79	Bláha e Maršálek, 2001
Tailândia	1994	0,07 - 0,35	-	-	Citado em Sivonen e Jones, 1999

Por outro lado, e, como se referiu em 2.4, a ingestão continuada de 50 ng.L^{-1} poderá estar associada a um aumento da incidência de cancro do cólon. Considerando os valores da tabela 4, a exposição humana a níveis de microcistinas similares é um

cenário real, em locais onde os *blooms* são frequentes e onde o tratamento de água é muito rudimentar.

A informação acerca do teor de microcistinas na água tratada é bastante restrita e resume-se, fundamentalmente, a dados obtidos em estudos de eficácia dos sistemas de tratamento de água. Porém, a informação disponível sugere que os métodos convencionais de tratamento de água, incluindo a utilização de carbono activado, não são totalmente eficazes na remoção de microcistinas (Duy et al., 2000). Por outro lado, nalguns países o tratamento da água bruta resume-se apenas à cloragem, não se efectuando os processos de filtração e adsorção e, inclusivamente, há populações que consomem água não tratada (Duy et al., 2000). Um estudo efectuado em 1995 em 160 reservatórios de água doce no Canadá, revelou que 68% das amostras de água tratada provenientes de água bruta contaminada com microcistinas continha toxinas (Gupta et al., 2001). O episódio da unidade de hemodiálise de Caruaru revelou que os pacientes estiveram expostos a 19,5 $\mu g.L^{-1}$ de microcistina e que o processo de tratamento da água na origem não foi adequado. Contudo, se admitíssemos que o tratamento tenha removido 99% das toxinas, o valor na água bruta seria de 1.900 $\mu g.L^{-1}$ de microcistina, valor perfeitamente espectável, de acordo com os níveis máximos detectados em alguns países.

De um modo geral, e considerando que o tratamento da água é adequado, sobretudo se for empregue a ozonização, a exposição humana a microcistinas através da água de consumo não excederá o valor de 1 $\mu g.L^{1}$ (Duy et al., 2000;WHO, 2003). Porém, embora o tratamento da água bruta possa salvaguardar a ocorrência de episódios de intoxicação humana aguda, está ainda por esclarecer o impacto na saúde humana decorrente da ingestão prolongada de doses inferiores.

3.3. Regulamentação do teor de microcistinas na água – o valor guia da OMS

Em 1998 a Organização Mundial de Saúde estabeleceu como valor-guia para o teor de microcistinas em água de consumo o valor de 1 $\mu g.L^{-1}$ (1 nM), expresso em equivalentes da MCLR (WHO, 1998). Este valor representa a concentração máxima de microcistinas cujo consumo, durante todo o período de vida dos indivíduos, não acarreta qualquer risco para a saúde humana.

A determinação do valor-guia para a MCLR (VG_{MCLR}) baseou-se num estudo sub-crónico com murganhos (15 machos e 15 fêmeas) administrados oralmente durante 13 semanas com MCLR (40, 200 e 1000 $\mu g.Kg_{pc}^{-1}.dia^{-1}$) (WHO, 1998). Os animais tratados com a dose mais baixa (40 $\mu g.Kg^{-1}$) não apresentaram quaisquer sinais de patologia hepática. A dose de 200 $\mu g.Kg^{-1}$ induziu apenas patologia ligeira em alguns animais. A maioria dos animais administrados com a dose mais elevada (1000 $\mu g.Kg^{-1}$) apresentou alterações hepáticas tais como inflamação crónica, degeneração hepática e depósitos de hemosiderina (complexo de ferro cuja deposição em órgãos é um processo característico de situações hemorrágicas). Para as duas doses mais elevadas (nos machos) registou-se um aumento significativo dos níveis séricos de transaminases, a redução do nível de gama glutamil transferase, proteína total e albumina. As fêmeas apresentaram alterações nas transaminases apenas à dose mais elevada. Para os animais de ambos os sexos tratados com 1000 $\mu g.Kg^{-1}$ observou-se o aumento do consumo de alimentos (entre 14-20%), mas a redução do peso corporal em 7%. A concentração de 40 $\mu g.Kg^{-1}$ foi considerada como a dose de MCLR que não induz efeitos nocivos (WHO, 1998).

O valor guia foi calculado de acordo com a fórmula:

$$\mathbf{VG_{MCLR} = (DDA \times pc \times fa) / v} \qquad \text{com} \qquad \mathbf{DDA = NOAEL / fi}$$

Em que:

DDA = Dose diária aceitável ($\mu g.Kg_{pc}^{-1}.dia^{-1}$);

pc = peso corporal (60 Kg, para um adulto);

fa = factor de alocação (considerou-se que 80% da exposição humana a microcistinas ocorre através da água de consumo);

v = volume de água consumido diariamente (2L, para um adulto);

NOAEL = dose que não causa efeito nocivo (*No Observed Adverse Effects Level*, $\mu g.Kg^{-1}$);

fi = factor de incerteza.

Com base no NOAEL de 40 $\mu g.Kg^{-1}$ e num factor de incerteza de 1000 (100 para variações intra e inter específicas e 10 para a limitação de informação sobre toxicidade crónica), determinou-se um valor de DDA de 0,04 $\mu g.kg_{pc}^{-1}.dia^{-1}$. Considerando o peso médio de um adulto de 60 Kg, o consumo de 2L de água por dia e um factor de alocação de 0,8, obteve-se o valor-guia de 0,96 $\mu g.L^{-1}$,

relativamente ao teor total de MCLR (extracelular + intracelular) em água de consumo. Este valor foi aproximado para 1 $\mu g.L^{-1}$ (WHO, 1998).

Este valor foi posteriormente confirmado através de um estudo sub-crónico com suínos (grupos de 5 animais) expostos através da água de beber a um extracto de *M. aeruginosa* contendo MCLR (184, 522 e 860 $\mu g.kg_{pc}^{-1}.dia^{-1}$) durante 44 dias (Duy et al., 2000). Para a dose mais baixa apenas um animal foi afectado, pelo que a dose de 184 $\mu g.kg^{-1}$ foi considerada como a dose mínima que causa efeito (LOAEL, *Lowest Observed Adverse Effect Level*). Aplicando o valor de LOAEL em vez do NOAEL na fórmula anterior e considerando um factor de incerteza de 5000 (100 para variações intra e interespecíficas, 5 pela utilização do LOAEL e 10 pelo tempo do estudo ser inferior ao tempo de vida dos animais), o valor-guia para adultos foi recalculado e determinou-se os valores-guia para crianças de 5 e 10 Kg (Duy et al., 2000), considerando um consumo de água de 0,75 L e 1 L, respectivamente (tabela 5). Estes autores calcularam, também, valores-guia para a MCLR tendo em conta a sua capacidade de promoção tumoral, usando para tal o valor de NOAEL e um factor de incerteza de 3000 (100 para variações intra e interespecíficas, 10 para o tempo de vida e 3 para a actividade de promoção tumoral), tendo obtido os valores indicados na tabela 5. Estes valores não vigoram, ainda, como valores-guia oficiais.

Tabela 5. Valores-guia para a MCLR em água de consumo (adaptado de Duy et al., 2000).

Grupos etários	Valores-guia ($\mu g.L^{-1}$)	
	Toxicidade aguda	Promoção tumoral
Bebés (5 Kg)	0,20	0,07
Crianças (10 Kg)	0,29	0,11
Adultos (60 Kg)	0,88 (similar ao VG_{MCLR} da OMS)	0,32

Alguns países adoptaram directamente o VG_{MCLR} da OMS para o seu direito interno como valor paramétrico de referência para as microcistinas em água de consumo: Brasil, Coreia, Espanha, França, Japão, Noruega, Nova Zelândia, Polónia e República Checa (Burch, 2008). Outros países como a Austrália ou Canadá, adoptaram o mesmo método de determinação do valor guia da OMS e aplicaram alguns requisitos locais, tendo obtido valores similares (Burch, 2008). Em Portugal este valor é também de 1 $\mu g.L^{-1}$ (Decreto-Lei 306/2007 de 27 de Agosto).

Para massas de água destinadas a actividades recreativas a OMS estabeleceu três níveis de alerta relativamente à densidade cianobacteriana (Chorus et al., 2000): um nível de risco reduzido (≤ 20.000 células.mL^{-1}); um nível de risco moderado (20.000 – 100.000 células.mL^{-1}); um nível de risco elevado (> 100.000 células.mL^{-1}). Estes valores foram determinados com base no valor-guia das microcistinas para água de consumo e no teor intracelular máximo de microcistinas na célula cianobacteriana (0,2 pg), pelo que correspondem a potenciais concentrações de microcistinas de 2-4 μg.L^{-1}, 10-20 μg.L^{-1} e >1 mg.L^{-1}, respectivamente (Codd et al., 2005). Estes níveis de alerta constituem uma ferramenta útil para as autoridades de saúde e para as entidades gestoras dos recursos hídricos uma vez que permitem instituir algumas medidas preventivas do risco de exposição a microcistinas, tais como a proibição temporária de utilização dos reservatórios.

No entanto, o VG_{MCLR} e, consequentemente, os níveis de alerta, apresentam limitações em termos de protecção da saúde pública. Por um lado, foram determinados com base, apenas, em dados de hepatotoxicidade aguda, não contemplando efeitos decorrentes da exposição prolongada a baixas doses, designadamente efeitos cancerigénicos. A esta restrição somam-se as limitações analíticas. Como se referiu em 3.1, os limites de detecção dos métodos mais usados na monitorização de microcistinas são da mesma ordem de grandeza (HPLC) ou uma ordem de grandeza abaixo (ELISA) do VG_{MCLR} e, portanto, as metodologias poderão não detectar doses que induzem eventuais efeitos sub-agudos e crónicos. Por outro lado, o valor-guia e os níveis de alerta não incluem potenciais efeitos noutros órgãos nem, consequentemente, a toxicidade total no organismo.

Saliente-se ainda que não foram instituídos valores-guia para outras cianotoxinas. Exceptua-se apenas o Brasil, que estabeleceu recomendações para a cilindrospermopsina (15 μg.L^{-1}) e saxitoxina (3 μg.L^{-1}) (Burch, 2008). Porém, tal como para as microcistinas, evidências crescentes apontam para uma eventual acção genotóxica da nodularina (Lankoff et al., 2006c) e da cilindrospermopsina (Humpage et al., 2000b), que são produzidas por espécies que co-ocorrem frequentemente com as espécies produtoras de microcistinas. Assim, as populações poderão estar expostas a baixas doses de uma mistura de potenciais genotoxinas cianobacterianas. Desconhece-se se os efeitos das várias toxinas são cumulativos, sinergísticos ou antagónicos, mas, de

qualquer forma, este risco supostamente acrescido de exposição humana a misturas de cianotoxinas não foi contemplado em qualquer valor guia ou recomendação.

Embora seja cada vez mais evidente que as microcistinas se propagam/acumulam na cadeia alimentar (Vasconcelos 2001a,b; Cood et al., 2005; Wiegand e Pflugmacher, 2005; Ibelings e Chorus, 2007), não é possível, à luz do conhecimento actual, estabelecer quaisquer valores guia ou de alerta para actividades como a produção agrícola e a pesca (Burch, 2008).

Tal como foi referido anteriormente, ainda não foi desenvolvido um método simultaneamente robusto, rápido, específico e económico que permita a identificação e quantificação de microcistinas, um requisito fundamental para a monitorização ambiental e biomonitorização de populações. Como tal, não é ainda possível regulamentar com rigor os níveis de microcistina no ambiente nem tão pouco proceder a uma avaliação do risco rigorosa da exposição humana a microcistinas.

4. Objectivos

Objectivo principal

Atendendo a que a exposição crónica a concentrações vestigiais de microcistinas é a forma de exposição humana mais provável e face à hipótese de que esta exposição poderá estar associada ao desenvolvimento de cancro, o trabalho conducente à presente dissertação teve como principal objectivo a avaliação do potencial cancerigénico de microcistinas.

Os resultados experimentais obtidos através de várias abordagens metodológicas foram estruturados na forma de seis artigos (publicados ou em fase de submissão) que constituem os capítulos 2 a 6 e cujos objectivos específicos se enunciam seguidamente:

Objectivos específicos

Capítulo 2

- Selecção de um modelo celular *in vitro* sensível à microcistina-LR (MCLR), através do estudo da citotoxicidade de extractos cianobacterianos em três linhas celulares de mamífero: hepatócitos humanos (HepG2), hepatócitos de murganho (AML12) e células epiteliais de rim de macaco (Vero-E6);
- Estudo preliminar da genotoxicidade de extractos cianobacterianos no modelo previamente seleccionado (Vero-E6).

Capítulo 3

- Confirmação da toxicidade da MCLR no modelo celular Vero-E6 através da comparação dos efeitos citotóxicos de extractos cianobacterianos e de toxina pura (comercial).
- Determinação do limiar de citotoxicidade.

Capítulo 4

- Identificação dos alvos intracelulares da MCLR na linha celular Vero-E6.
- Caracterização dos efeitos da MCLR ao nível intracelular.

Capítulo 5

- Estudo dos efeitos genotóxicos da MCLR no modelo celular Vero-E6.
- Comparação com o modelo de hepatócitos HepG2.

Capítulo 6

- Análise do efeito da MCLR nas vias de activação de sinais mitogénicos ERK1/2, p38 e JNK no modelo celular Vero-E6.
- Avaliação do efeito da MCLR na proliferação celular.

Referências bibliográficas

Andrinolo, D., Sedan, D., Telese, L., Aura, C., Masera, S., Giannuzzi, L., Marra, C.A., Alaniz, M.J.T. 2008. Hepatic recovery after damage produced by sub-chronic intoxication with the cyanotoxin microcystin LR. Toxicon 51: 457-467.

Apeldoorn, M.E., Egmond, H.P., Speijers, G.J.A., Bakker, G.J.I. 2007. Toxins of cyanobacteria. Mol. Nutr.Food Res. 51: 7-60.

Batista, T., De Sousa, G., Suput, J.S., Rahmani, R., Šuput, D. 2003. Microcystin-LR causes the collapse of actin filaments in primary human hepatocytes. Aquat. Toxicol. 65: 85-91.

Billam, M., Mukhi, S., Tang, L., Weimin, G., Wang, J.S. 2008. Toxic response indicators of microcystin-LR in F344 rats following a single dose treatment. Toxicon 51: 1068-1080.

Bischoff, K. 2001. The toxicology of microcystin-LR: occurrence, toxicokinetics, toxicodynamics, diagnosis and treatment. Vet. Human Toxicol. 43: 294-297.

Bláha, L., Maršálek, B. 2001. Dissolved microcistins in raw and treated drinking water in the Czech republic. In: I. Chorus (Ed.) Cyanotoxins – occurrence, causes and consequences, Springer Publishers, Heidelberg, pp. 212-216.

Boelsterli, U.A. 2009. Mechanistic toxicology: the molecular basis of how chemicals disrupt biologigal targets, Informa Healthcare USA, Inc., New York, pp.233.

Botha, N., Van de Venter, M., Downing, T.G., Shepard, E.G., Gehringer, E.G. 2004. The effect of intraperitoneally administered microcystin-LR on the gastrointestinal tract of Balb/c mice. Toxicon 43: 251-254.

Bouaïcha, N., Maatouk, I., 2004. Microcystin-LR and nodularin induce intracellular glutathione alteration, reactive oxygen species production and lipid peroxidation in primary cultured hepatocytes. Toxicol. Lett. 148, 53-63.

Bouaïcha, N., Maatouk, I., Plessis, M.J., Périn, F. 2005. Genotoxic potential of microcystin-LR and nodularin *in vitro* in primary cultured rat hepatocytes and *in vivo* rat liver. Environ. Toxicol. 20: 341-347.

Boaru, D.A., Dragoş, N., Schrimer, K., 2006. Microcystin-LR induced cellular effects in mammalian and fish primary hepatocyte cultures cell lines: A comparative study. Toxicology 218, 134-148.

Bolt, H.M., Foth, H., Hensgtler, J.G., Degan, G.H. 2004. Carcinogenecity categorization of chemicals – new aspects to be considered in a European perspective.Toxicology Letters 151: 29-41.

Burch, M.D. 2008. Effective doses, guidelines & regulations. Adv Exp Med Biol. 619: 831-53.

Chang, L., Karin, M. 2001. Mammalian MAP kinases signalling cascades. Nature 410: 37-40.

Chen, T., Qingsong, W., Cui, J., Yang, W., Shi, Q., Hua, Z., Ji, J., Shen, P. 2005. Induction of apoptosis in mouse liver by microcystin-LR. Mol. Cell. Proteo. 4: 958-974.

Chen, Y.M., Lee, T.H., Lee, S.J., Huang, H.B., Huang, R.H., Chou, H.N. 2006. Comparison of protein phosphatase inhibition activities and mouse toxicities of microcystins. Toxicon 47: 742-764.

Chorus, I., Falconer, I.R., Salas, H.J., Bartram, J. 2000. Health risks caused by freshwater cyanobacteria in recreational waters. J. Toxicol. Environ. Health, Part B 3: 323-347.

Chorus, I. 2001. Cyanotoxin occurrence in freshwaters – a summary of survey results from different countries. In: I. Chorus (Ed.) Cyanotoxins – occurrence, causes and consequences, Springer Publishers, Heidelberg, pp. 75-82.

Chorus, I., Fastner, J. 2001. Recreational exposure to cyanotoxins. In: I. Chorus (Ed.) Cyanotoxins – occurrence, causes and consequences, Springer Publishers, Heidelberg, pp. 190-199.

Chorus, I., Heinze, R., Hübner, C., Schmidt, W., Pietch, J. 2001. Elimination of microcystins at Dörtendorf: conventional treatment and a pilot experimental treatment system. In: I. Chorus (Ed.) Cyanotoxins – occurrence, causes and consequences, Springer Publishers, Heidelberg, pp. 221-225.

Clark, S.P., Ryan, T.P., Seafoss, G.H., Davis, M.A., Hooser, S. 2008. Chronic microcystin exposure induces hepatocyte proliferation with increased expression of mitotic and cyclin-associated genes in p53-deficient mice. Toxicol. Phatol. 36, 190-203.

Codd, G.A. 2000. Cyanobacterial toxins, the perception of water quality, and the priorization of eutrophication control. Ecol. Eng. 16: 51-60.

Codd, G.A., Morrison, L.F., Metcalf, J.S. 2005. Cyanobacterial toxins: risk management for health protection. Toxicol. Appl. Pharmacol. 203: 264-272.

Dahlem, A.M., Hassan, A.S., Swanson, S.P., Carmichael, W.W., Beasley, V.R. 1989. A model system for studying the bioavailability of intestinally administered microcystin-LR, a hepatotoxic peptide from the cyanobacterium *Microcystis aeruginosa*. Pharmacol. Toxicol. 64: 177-181.

Dawson, R.M. 1998. The toxicology of microcystins. Toxicon 36:953-962.

Dawson, J.F., Holmes, C.F.B. 1999. Molecular mechanisms underlying inhibition of protein phosphatases by marine toxins. Frontiers in Bioscience 4, d646-658.

Dietrich, D., Hoeger, S. 2005. Guidance values for microcystins in water and cyanobacterial supplement products (blue-green algal supplements): a reasonable or misguided approach? Toxico. Appl. Pharmacol. 203: 273-289.

Ding, W.X., Shen, H.M., Zhu, H.G., Lee, B.L., Ong, C.N. 1999. Genotoxicity of microcistic cyanobacterial extract of a water source in China, Mutat Res. 442: 69-77.

Ding, W-X., Shen, H-M., Ong, C-N. 2000a. Microcystic cyanobacteria extract induces cytoskeleton disruption and intracellular glutathione alteration in hepatocytes. Environ. Health Persp. 108: 605-609.

Ding, W-X., Shen, H-M., Ong, C-N., 2000b. Critical role of reactive oxygen species and mitochondrial permeability transition in microcystin-induces rapid apoptosis in rat hepatocytes. Hepatology 32, 547-555.

Ding, W-X., Shen, H-M., Ong, C-N. 2001. Pivotal role of mitochondrial Ca^{2+} in microcystin-induced mitochondrial permeability transition in rat hepatocytes. Biochem. Bioph. Res. Co. 285: 1155-1161.

Ding, W.X., Ong, C.N. 2003. Role of oxidative stress and mitochondrial changes in cyanobacteria-induced apoptosis and hepatotoxicity, FEMS Microbio. Lett. 10880: 1-7.

Ding, X.S., Li, X.Y., Duan, H.Y., Chung, I.K., Lee, J.A. 2006. Toxic effects of Microcystis cell extracts on the reproductive system of male mice. Toxicon 48: 973-979.

Duy, T.N., Lam, P.K.S., Shaw, G.R., Connell, D.W. 2000. Toxicology and risk assessment of freshwater cyanobacterial (blue-green algal) toxins in water, Rev. Environ. Contam. Toxicol. 163 : 113-186.

Eriksson, J.E., Toivola, D., Meriluoto, J.A.O., Karaki, H., Han, Y-G., Hartshorne, D. 1990. Hepatocyte deformation induced by cyanobacterial toxins reflects inhibition of protein phosphatases. Biochem. Biophys. Res. 171: 1347-1353.

Falconer, I.R. 1991. Tumour promotion and liver injury caused by oral consumption of cyanobacteria, Environ. Toxicol. Water Qual. 6: 177-184.

Falconer, I.R., Yeung, D.S. 1992. Cytoskeletal changes in hepatocytes induced by microcystis toxins and their relation to hyperphosphorilation of cell proteins, Chem. Biol. Interact. 81: 181-196.

Fastner, J., Wirsing, B., Wiedner, C., Heinze, R., Neumann, U., Chorus, I.. 2001. Cyanotoxin occurrence in German. Microcystins and hepatocyte toxicity. In: I. Chorus (Ed.) Cyanotoxins – occurrence, causes and consequences, Springer Publishers, Heidelberg, pp. 22-36.

Fawell, J.K., Hart, J., James, H.A., Parr, W. 1993. Blue-green algae and their toxins — analysis, toxicity, treatmentand environmental control. Water supply 11:109-121.

Fawell, J.K.1999. The toxicity of cyanobacterial toxins in the mouse: I Microcystin-LR. Human Exp. Toxicol. 18: 162-167.

Fenech, M. 2000. The in vitro micronucleus technique. Mut. Res. 455: 81-95.

Fessard, V., Le Hegarat, L., Mourot, A. 2004. Comparison of the genotoxic results obtained from the in vitro cytokinesis-block micronucleous assay with various toxins inhibitors of protein phosphatases: okadaic acid, nodularin and microcystin-LR, in: Proceedings of Sixth International Conference on Toxic Cyanobacteria, Bergen, Norway 21-27 June, pp.68-69.

Fischer, W.L, Altheimer, S., Cattori, V., Meier, P.J., Dietrich, D.R., Hagenbuch, B. 2005. Organic anion transporting polypeptides expressed in liver and brain mediate uptake of microcystin. Toxicol. Appl. Pharmacol. 203: 257-263.

Fladmark, K.E., Brustugun, O.T., Hovland, R., Bøe, R., Gjertsen, B.T., Zhivotovsky, B., Døskeland, S.O., 1999. Ultrarapid caspase-3 dependent apoptosis induction by serine/threonine phosphatise inhibitors. Cell Death Differ. 6, 1099-1108.

Fladmark, K.E., Brustugun, O.T., Mellgren, G., Krakstad, C., Bøe, R., Vintermyr, O.K., Schulman, H., Døskeland, S.O., 2002. Ca^{2+}/Calmodulin-dependent protein kinase II is required for microcystin-induced apoptosis. J. Biol. Chem. 277, 2804-2811.

Frangež, R., Žužek, M.C., Mrkun, J., Šuput, D., Sedmak, B., Kosec, M., 2003. Microcystin-LR affects cytoskeleton and morphology of rabbit primary whole embryo cultured cells in vitro. Toxicon 41: 999-1005.

Fu, W. Chen, J., Wang, X., Xu, L. 2005. Altered expression of p53, Bcl-2 and Bax induced by microcystin-LR in vivo and in vitro. Toxicon 46, 171-177.

Funari, E., Testai, E. 2008. Human health risk assessment related to cyanotoxins exposure. Critical Rev. Toxicol. 38: 97-125.

Galvão, H.M., Reis, M.P., Valério, E., Domingues, R.B, Costa, C, Lourenço, D., Condinho, S., Miguel, R., Barbosa, A., Gago, C., Faria, N., Paulino, S., Pereira, P. 2008. Cyanobacterial blooms in natural waters in southern Portugal: a water managmente perspective. Aquat. Microbiol. Ecol. 53: 129-140.

Gaudin, J., Huet, S., Jarry, G., Fessard, V. 2008a. *In vivo* DNA damage induced by the cyanotoxin microcystin-LR: comparison of intra-peritonial and oral administration by use of the comet assay. Mutat. Res. 652: 65-71.

Gaudin, J., Hegarat, L., Nesslany, F., Marzin, D., Fessard, V. 2008b. *In vivo* genotoxic potential of microcystin-LR: a cyanobacterial toxin, investigated bith by the unscheduled DNA synthesis (UDS) and the comet assays after intravenous administration. Environ. Toxicol. 24: 200-209.

Gehringer, M.M. 2004. Microcystin-LR and okadaic acid-induced cellular effects: a dualistic response. FEBS Letters 557, 1-8.

Grabow, W., Randt, W., Prozesky, O., Scott, W. 1982. *Microcystis aeruginosa* toxin: cell culture toxicity, hemolysis and mutagenicity assays, Appl. Environ. Microbiol. 43: 1425-1433.

Graham, J.M., Wicox, L.W. 2000. Cyanobacteria. In: Graham, J.M., Wilcox, L.W. (Eds.) Algae, Prentice-Hall, N.Y., pp.97-131.

Gupta, S., Giddings, M., Sheffer, M.. 2001. Cyanobacterial toxins in drinking water: a Canadian perspective. In: I. Chorus (Ed.) Cyanotoxins – occurrence, causes and consequences, Springer Publishers, Heidelberg, pp. 208-212.

Gupta, N., Pant, S.C., Vijayaraghavan, R., Rao, P.V.L. 2003. Comparative toxicity evaluation of cyanobacterial cyclic peptide toxin microcystin variants (LR, RR, YR) in mice. Toxicology 188: 285-296.

Guzman, R.E., Solter, P.H., 1999. Hepatic oxidative stress following prolonged sublethal microcystin-LR exposure. Toxicol. Phatol. 27, 582-588.

Guzman, R.E., Solter, P.F., Runnegar, M.T. 2003. Inhibition of nuclear protein phosphatase activity in mouse hepatocytes by the cyanobacterial toxin microcystin-LR. Toxicon 41, 773-781.

Hagenbuch, B., Meier, P.J. 2003. The superfamily of organic anion transporting polypeptides, Biochimica et Biophysica Acta 1609: 1-18.

Harada, K.I. 1996. Chemistry and detection of microcystins. In: M.F. Watanabe, K.I. Harada, W.W. Carmicahel, H. Fujiki (Eds.) Toxic *microcystis*, CRC Press, New York, pp.103-148.

Hernández, J.M., López-Rodas, V., Costas, E. 2009. Microcystins from tap water could be a risk factor for liver and colorectal cancer: A risk intensified by global change. Med. Hypoth.72:539-540.

Honkanen, R.E., Zwiller, J., Moore, R.E., Daily, S.L., Khatra, B.S., Dukelow, M., Boynton, A.L. 1990. Characterization of microcystin-LR, a potent inhibitor of type 1 and type 2A protein phosphatases, J. Biol. Chem. 265: 19401-19404.

Hooser, S.B., 2000. Fulminant hepatocyte apoptosis in vivo following microcystin-LR administration to rats. Toxicol. Phatol. 278, 726-733.

Humpage, A.R., Hardy, S.J., Moore, E.J., Froscio, S.M., Falconer, I.R. 2000a. Microcystins (cyanobacterial toxins) in drinking water enhance the growth of aberrant crypt foci in the mouse colon. J. Toxicol Environm Health A 61: 155-65.

Humpage, A.R., Fenech, M., Thomas, P., Falconer, I.R. 2000b. Micronucleous induction and chromosome loss in transformed human white cells indicate clastogenic and aneugenic action of the cyanobacterial toxin, cylindrospermopsin. Mutat. Res. 472: 155-161.

IARC. 2006. Cyanobacterial peptide toxins. Available at: http://monographs.iarc.fr/ENG/Meetings/94-cyanobacteria.pdf.

Iarmarcovai, G., Botta, A., Orsière, T. 2006. Number of centromeric signals in micronuclei and mechansims of aneuploidy. Toxicology Letters 166: 1-10.

Ibelings, B.W., Chorus, I. 2007. Accumulation of cyanobacterial toxins in freshwater "seafood" and its consequences for public health: a review. Envir. Pollution 150: 177-192.

ISO 20179, Water quality – determination of microcystins – method using solid phase extraction (SPE) and high performance liquid chromatography (HPLC) with ultraviolet (UV) detection.

Ito, E., Kondo, F., Harada, K.I. 1997a. Hepatic necrosis in aged mice by oral administration of microcystin-LR. Toxicon 35: 231-239.

Ito, E., Kondo, F., Terao, K., Harada, K.I. 1997b. Neoplastic nodular formation in mouse liver induced by repeated intraperitoneal injection of microcystin-LR, Toxicon 35: 1453-1457.

Ito, E., Kondo, F., Harada, K.I. 2001. Intratracheal administration of microcystin-LR, and its distribution. Toxicon 39: 265-271.

Ito, E., Takai, A., Kondo, F., Masui, H., Imanishi, S., Harada, K.I. 2002. Comparison of protein phosphatase inhibitory activity and apparent toxicity of microcystins and related compounds. Toxicon 40: 1017-1025.

Jones, G.J., Orr, P.T.1994. Release and degradation of microcystin following algicide treatment of a *Microcystis aeruginosa* bloom in a recreational lake, as determined by HPLC and protein phosphatase inhibition assay. Water research 28: 871-876.

Junttila, M.R., Li, S.P., Westermarck, J. 2008. Phosphatase-mediated crosstalk between MAPK signaling pathways in the regulation of cell survival. FASEB J. 22: 954-965.

Kenefick, S.L., Hrudey, S.E., Peterson, H.G., Prepas, E.E. 1993. Toxin release from *Microcystis aeruginosa* after chemical treatment. Water Sci. Technol. 27: 433-440.

Khan, S.A., Ghosh, S., Wickstrom, M., Miller, L.A., Hess, R., Haschek, W.M., Beasley, V.R. 1995. Comparative pathology of microcystin-LR in cultured hepatocytes, fibroblasts and renal epithelial cells. Nat. Toxins 3: 119-128.

Khan, S.A,. Wickstrom, M.L, Haschek, W.M., Schaeffer, D.J., Ghosh, S., Beasley, V.R. 1996. Microcystin-LR and kinetics of cytoskeletal reorganization in hepatocytes, kidney cells and fibroblasts. Nat. Toxins 4: 206-214.

Kirsch-Volders, M., Vanhauwaert, A., De Boeck, M., Decordier, I. 2002. Importance of detecting numerical versus structural chromosome aberrations. Mut. Res. 504: 137-148.

Kolch, W. 2000. Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. Biochem.J. 351:289-305.

Kondo, F., Matsumoto, H., Yamada, S., Ishikawa, N., Ito, E., Nagata, S., Ueno, Y., Suzuki, M., Harada, K. 1996. Detection and identification of metabolites of microcystins formed in vivo in mouse and rat livers. Chem. Res. Toxicol. 9: 1355-1359.

Komatsu, M., Furukawa, T., Ikeda, R., Takumi, S., Nong, Q., Aoyama, K., Akiyama, S., Keppler, D., Takeuchi, T. 2007. Involvement of mitogen-activated protein kinase signaling pathways in microcystin-LR-induced apoptosis after its selective uptake mediated by OATP1B1 and OATP1B3. Toxicol. Sci. 97: 407-416.

Kruschwitz, C., Chorus, I., Heize, R., Schlag, G., Gröbe, K. 2001. Elimination of microcistins in the Rostock drinking-water treatment plant. In: I. Chorus (Ed.) Cyanotoxins – occurrence, causes and consequences, Springer Publishers, Heidelberg, pp. 217-221.

Kuiper-Goodman, T., Falconer, I, Fitzgerald, J. 1999. Human health aspects. In: I. Chorus, J. Bartram (Eds.) Toxic cyanobacteria in water: a guide to their public health, consequences, monitoring and managment, E & FN Spon, London, pp. 113-153.

Laconi, E. 2007. The evolving concept of tumour microenvironments. BioEssays 29: 738-744.

Lankoff A., Banasik, A., Obe, G., Deperas, M., Kuzminski, K., Tarczynska, M., Jurczak, T., Wojcik, A. 2003. Effect on microcystin-LR and cyanobacterial extract from Polish reservoir of drinking water on cell cycle progression, mitotic spindle, and apoptosis in CHO-K1 cells. Toxicol. Appl. Pharm. 189: 204-213.

Lankoff, A., Krzowski, L., Glab, J., Banasik, A., Lisowska, H., Kuszwski, T., Gozdz, S., Wojcik, A. 2004. DNA damage and repair in human peripheral blood lymphocytes following treatment with microcystin-LR. Mut. Res. 559: 131-142.

Lankoff, A., Bialczyki, J., Dziga, D., Carmichael, W.W., Lisowska, H., Wojcik, A. 2006a. Inhibition of nucleotide excision repair by microcystin-LR in CHO-K1 cells. Toxicon 48: 957-965.

Lankoff, A., Bialczyki, J., Dziga, D., Carmichael, W.W., Gradzka, I., Lisowska, H., Kuszewski, T., Gozdz, S., Piorun, I., Wojcik, A. 2006b. The repair of gamma-radiation-induced DNA damage is inhibited by microcystin-LR, the PP1 and PP2A phosphatase inhibitor. Mutagenesis 21: 83-90.

Lankoff, A., Wojcik, A., Fessard, V., Meriluoto, J. 2006c. Nodularin-induced genotoxicity following oxidative DNA damage and aneuploidy in HepG2 cells. Toxicology Letters 164: 239-248.

Li, X.Y., Liu, Y.D., Song, L.R. 2001. Cytological alterations in isolated hepatocytes from common carp (*Cyprinus carpio* L.) exposed to microcystin-LR. Inc. Environ. Toxicol. 16 : 517-522.

Li, L., Xie, P., Chen, J., 2005. In vivo studies on toxin accumulation in liver and ultrastructural changes in hepatocytes of the phytoplanktivorours bighead carp i.p. injected with extracted microcystins. Toxicon 46, 533-545.

Li, L., Xie, P., Chen, J., 2007. Biochemical and ultrastructural changes of the liver and kidney of the phytoplanktivorours silver carp feeding naturally on toxic *Microcystis* blooms in Taihu Lake, China. Toxicon 49, 1042-1053.

Li, H., Xie, P., Li, G., Hao, L., Xiong, Q. 2009. *In vivo* study on the effects of microcystin extracts on the expression profiles of proto-oncogenes (*c-fos*, *c-jun* and *c-myc*) in liver, kidney and testis of male Wistar rats injected i.v. with toxins. Toxicon 53: 169-175.

Lian, M., Liu, Y., Yu, S.Z., Qian, G.S., Wan, S.G., Dixon, K.R. 2006. Hepatitis B virus x gene and cyanobacterial toxins promote aflatoxin B_1-induced hepatotumorigenesis in mice. World J. Gastroenterol. 12: 3065-3072.

MacKintosh, R.W., Dalby, K.N., Campbell, D.G., Cohen, P.T.W., Cohen, P., Mackintosh, C. 1995. The cyanobacterial toxin microcystin binds covalently to cysteine-273 on protein phosphatase 1. Febs Lett. 371: 236-240.

Maatouk, I., Bouaïcha, N., Plessis, M.J., Périn, F. 2004. Detection by 32P-postlabelling of 8-oxo-7,8-dihydro-2'-deoxyguanosine in DNA as biomarker of microcystin-LR- and nodularin-induced DNA damage *in vitro in vitro* in primary cultured rat hepatocytes and *in vivo* in rat liver, Mut. Res. 564: 9-20.

Maidana, M., Carlis, V., Galhardi, F.G., Yunes, J.S., Geracitano, L.A., Monserrat, J.M., Barros, D.M. 2006. Effects of microcystins over short- and long-term memory and oxidative stress generation in hippocampus of rats. Chemico-Biological Interactions 159: 223-234.

Mankiewicz, J., Tarczynska, M., Fladmark, K.E., Doskeland, S.O., Walter, Z., Zalewski, M. 2001. Apoptotic effect of cyanobacterial extract on rat hepatocytes and human lymphocytes. Environ. Toxicol. 16: 225-233.

Mankiewicz, J., Walter, Z., Tarczynska, M., Palyvoda, O., Staniaszczyk, M., Zalewski, M. 2002. Genotoxicity of cyanobacterial extracts containing microcystins from Polish water reservoirs as determined by SOS Chromotest and Comet Assay. Environ. Toxicol. 17: 341-350.

McDermott, C.M., Nho, C.W., Howard, W., Holton, B. 1998. The cyanobacterial toxin, microcystin-LR, can induce apoptosis in a variety of cell types. Toxicon 36: 1981-1996.

McKay, M.M., Morrison, D.K. 2007. Integrating signals from RTKs to ERK/MAPK. Oncogene 26: 3113-3121.

Meloche, S., Pouysségur, J. 2007. The ERK1/2 mitogen-activated +rptein kinase pathway as a mastare regulator of the G1- to S-phase transition. Oncogene 26: 3227-3239.

Meriluoto J.A., Nygård S.E., Dahlem A.M., Eriksson J.E. 1990. Synthesis, organotropism and hepatocellular uptake of two tritium-labeled epimers of dihydromicrocystin-LR, a cyanobacterial peptide toxin analog. Toxicon 28: 1439-1446.

Milutinović, A., Sedmark, B., Horvat-ŽnidarŠić, I., Šuput, D. 2002. Renal injuries induced by chronic intoxication with microcystins. Cell. Mol. Biol. Lett. 7: 139-141.

Milutinović, A., Živin, M., Zorc-Plesković, R., Sedmark, B., Šuput, D. 2003. Nephrotoxic effects of chronic administration of microcystins-LR and –YR. Toxicon 42: 281-288.

Moreno, I., Repetto, G., Cameán, A.M. 2003. Interés toxicológico de las microcistinas. Ver. Toxicol 20: 159-165.

Moreno, I., Pichardo, S., Jos, A., Gómez-Amores, L., Mate, A., Vazquez, C.M., Cameán, A.M. 2005. Antioxidant enzyme activity and lipid peroxidation in liver and kidney of rats exposed to microcystin-LR administered intraperitoneally. Toxicon 45: 395-402.

Mur, L.R., Skulberg, O.M., Utkilen, H. 1999. Cianobacteria in the environment. In: I. Chorus, J.Bartram (Eds.) Toxic cyanobacteria in water: a guide to their public health consequences, mpnitoring and management, E & FN Spon, London, pp.15-40.

Nishiwaki-Matsushima, R., Nishiwaki, S., Ohta T., Yoshizawa, S., Suganuma, M., Harada, K., Watanabe, M.F., Fujiki, H. 1991. Structure-function relationships of microcystins, liver tumor promoters, in interaction with protein phosphatase. Jpn J. Cancer Res. 82: 993-966.

Nishiwaki-Matsushima, R., Ohta, T., Nishiwaki, S., Suganuma, M., Kohyama, K., Ishikawa, T., Carmichael, W.W., Fujiki, H. 1992. Liver tumor promotion by the cyanobacterial cyclic peptide toxin microcystin-LR. J. Cancer Res. Clin. Oncol. 118: 420–424.

Nobre, A.C.L., Jorge, M.C.M., Menezes, D.B., Fonteles, M.C., Monteiro, H.S.A. 1999. Effects of microcystin-LR in isolated perfused rat kidney. Brazilian Journal of Medical and Biological Research 32: 985-988.

Nong, Q., Komatsu, M., Izumo, K., Indo, H.P., Xu, B., Aoyama, K., Majima, H.J., Horiuchi, M., Morimoto, K., Takeuchi, T. 2007. Involvement of reactive oxigen species in microcystin-LR-induced cytogenotoxicity. Free Radical Res. 41: 1326-1337.

Park, H.D. 2001. Cyanotoxins and cyanobacterial blooms in South Korean lakes. In: I. Chorus (Ed.) Cyanotoxins – occurrence, causes and consequences, Springer Publishers, Heidelberg, pp. 68-75.

Paulino, S., Valério, E., Faria, N., Fastner, J., Welker, M., Tenreiro, R., Pereira, P. 2009. Detection of Planktothrix rebescens (cyanobacteria) associated with microcystin production in a freshwater reservoir. Hydrobiologia 621: 207-211.

Pereira, P., Onodera, H, Andrinolo, D., Franca, S., Araújo, F., Lagos, N., Oshima, Y.2001. Co-occurrence of PSP toxins and microcystins in Montargil freshwater reservoir, Portugal. In: G.M. Hallegraeff, C.J. Bolch, R.J. Lewis (Eds.) Harmful Algal Blooms 2000, IOC, UNESCO, pp. 108-111.

Pflugmacher, S., Wiegand, C., Oberemm, A., Beattie, K.A., Krause, E., Codd , G.A., Steinberg, C.E.W.1998. Identification of an enzymatically formed glutathione conjugate of the cyanobacterial hepatotoxin microcystin-LR: the first step of detoxication. Biochem. Biophys. Acta 1425: 527-533.

Pflugmacher, S., Wiegand, C. 2001. Metabolism of microcystin-LR in aquatic organisms. . In: I. Chorus (Ed.) Cyanotoxins – occurrence, causes and consequences, Springer Publishers, Heidelberg, pp. 257.260.

Pichardo, S., Jos, A., Zurita, J.L, Salguero, M., Camean, A.M., Reppeto, G. 2007. The use of the fish cell lines RTG-2 and PLHC-1 to compare the toxic effects produced by microcystins LR and RR. *Toxicology in vitro* 19: 865-873.

Plancha, C.E., David-Ferreira, J.F. 1999. Citosqueleto - composição, organização e significado funcional In: C. Azevedo (Ed.) Biologia celular e molecular, LIDEL-Edições Técnicas, Lisboa, pp. 203-218.

Pouria, S., Andrade, A., Barbosa, J., Cavalcanti, R.L., Barreto, V.T.S., Ward, C.J., Preiser, W., Poon, G.K. 1998. Fatal microcystin intoxication in haemodialysis unit in Caruaru, Brazil. Lancet 352: 21-26.

Raman, M., Chen, W., Cobb, M.H. 2007. Differential regulation and properties of MAPKs. Oncogene 26: 3100-3112.

Rao, P.V., Bhattacharya, R. 1996. The cyanobacterial toxin microcystin-LR induced DNA damage in mouse liver *in vivo.* Toxicology 114: 29-36.

Rao, P.V., Bhattacharya, R., Parida, M., Jana, A., Bhaskar, A. 1998. Freshwater cyanobacterium *Microcystis aeruginosa* (UTEX 2385) induced DNA damage *in vivo* and *in vitro*. Environ. Toxicol. Pharmacol. 5: 1-6.

Rao, P.V-L., Gupta, N., Jayaraj, R., Bhaskar, A.S.B., Jatav, P.C. 2005. Age-dependent effects on biochemical variables and toxicity induced by cyclic peptide toxin microcystin-LR in mice. Comp. Biochem. Physiol. 140:11-19.

Repavich, W.N., Sonzongni, J.H., Standridge, R.E., Wedphohl, L.F., Meisner, L.F. 1990. Cyanobacteria (blue-green algae) in Wisconsin waters: acute and chronic toxicity, Water Res. 24: 225-231.

Robinson N.A., Miura G.A., Matson C.F., Dinterman R.E., Pace J.G. 1989. Characterization of chemically tritiated microcystin-LR and its distribution in mice. Toxicon 27:1035-1042.

Robinson N.A., Pace J.G., Matson C.F., Miura G.A., Lawrence W.B. 1990. Tissue distribution, excretion and hepatic biotransformation of microcystin-LR in mice. J. Pharmacol.Exp. Ther. 256:176-182.

Rodríguez, E., Onstad, G.D., Kull, T.P.J., Metcalf, J.S., Acero, J.L., Gunten, U.V. 2007. Oxidative elimination of cyanotoxins: comparison of ozone, chlorine dioxide and permanganate. Water Res. 41: 3381-3393.

Runnegar M., Berndt N., Kong S.M., Lee E.Y.C., Zhang L. 1995. *In vivo* and *in vitro* binding of microcystin to protein phosphatases 1 and 2A. Biochem. Biophys. Res. Commun. 216: 162-169.

Saker, M.L., Nogueira, I.C.G., Vasconcelos, V.M., Neilan, B.A., Eaglesham, G.K., Pereira, P. 2003. First report and toxicological assessment of the cyanobacterium Cylindrospermopsis raciborskii from Portuguese freshwaters. Ecotoxicol. Environm. Safety 55: 243-250.

Sivonen, K., Jones, G. 1999. Cyanobacterial toxins. In: I. Chorus, J.Bartram (Eds.) Toxic cyanobacteria in water: a guide to their public health consequences, monitoring and management, E & FN Spon, London, pp.41-111.

Smith, J.L., Boyer, G.L., Zimba, P.V. 2008. A review of cyanobacterial odorous and bioactive metabolites: impacts and management alternatives in aquaculture. Aquaculture 208: 5-20.

Soares, M., Cagido, V.R., Ferraro, R.B., Meyer-Fernandes, J.R., Rocco, P.R.M., Zin, W.A., Azevedo, S.M.F.O. 2007. Effects of microcystin-LR on mouse lungs. Toxicon 50: 330-338.

Stotts, R.R., Twardock, A.R., Koritz, G.D., Haschek, W.M., Manuel, R.K., Hollis, W.B., Beasley, V.R. 1997a. Toxicokinetics of tritiated dihydromicrocystin-LR in swine. Toxicon 35: 455-465.

Stotts, R.R., Twardock, A.R., Koritz, G.D., Haschek, W.M., Choi, B.W., Rinehart, K.L., Beasley, V.R. 1997b. Distribution of tritiated dihydromicrocystin in swine. Toxicon 35: 937-953.

Suzuki, H., Watanabe, M.F., Wu, Y., Sugita, T., Kita, K., Sato, T., Wang, X., Tanzawa, H., Sekiya, S., Suzuki, N. 1998. Mutagenecity of microcystin-LR in human Rsa cells. Int. J. Mol. Med. 2: 109-112.

Teles, L.O., Pereira, E., Saker, M.,Vasconcelos, V. 2006. Time series forecasting of cyanobacteria blooms in the Crestuma reservoir (Douro river, Portugal) using artificial neural networks. Environmental Management 38: 227-237.

Toivola, D.M., Eriksson, J.E. 1999. Toxins affecting cell signaling and alteration of cytoskeletal structure. Toxicol. *In Vitro* 13: 521-530.

Tsuji, K., Naito, S., Kondo, F., Ishikawa, N., Watanabe, M.F., Suzuki, M., Harada, K.-I. 1994. Stability of microcystins from cyanobacteria: effect of light on decomposition et isomerization. Environ. Sci. Technol. 28: 173-177.

Tsuji, K., Watanuki, T., Kondo, F., Watanabe, M.F., Suzuki, S., Nakazawa, H., Suzuki, M., Uchida, H., Harada, K. 1995. Stability of microcystins from cyanobacteria-II. Effect of UV on decomposition and isomeration. Toxicon 33: 1619-1631.

Ueno, Y., Nagata, S., Tsutsumi, T., Hasegawa ,A., Watanabe, M.F., Park, H.D., Chen, G.C., Chen, G., Yu, S.Z. 1996. Detection of microcystins, a blue-green algal hepatotoxins, in drinking water sampled in Haimen and Fusui, endemic areas of primary liver cancer in China, by highly sensitive immunoassay. *Carcinogenesis* 17: 1317-1321.

Valério, E., Faria, N., Paulino, S., Pereira, P. 2008. Seasonal variation of phytoplankton and cyanobacteria composition and associated microcystins in six Portuguse freshwater reservoirs. Ann. Limnol.- Int. J. Limnol 44: 189 – 196.

Valério, E., Chambel, L., Faria, N., Paulino, S., Pereira, P., Tenreiro, R. 2009a. Molecular identification, typing and traceability of cyanobacteria from freshwater reservoirs. Microbiology 155: 642 – 656.

Valério, E. et al, Multiplex PCR for detection of microcystins-producing cyanobacteria from freshwater samples, Environ. Toxicol. (2009b), doi: 10.1002/Tox.20502.

Vasconcelos, V.M. 1993. Isolation of microcystin-LR from a Microcystis (cyanobacteria) waterbloom collected in the drinking water reservoir for Porto, Portugal. J. Environ. Sci. Health A28:2081-2094.

Vasconcelos, V.M. 1994. Toxic cyanobacteria (blue-green algae) in Portuguese freshwaters. Arch. Hydrobiol. 130:139-451.

Vasconcelos, V.M., Sivonen, K., Evans, W.R., Carmichael, W.W., Namikoshi, M. 1995. Isolation and characterization of microcystins (heptapeptide hepatotoxins) from Portuguese srains of Microcystis aeruginosa Kutz.emend Elekin. Arch. Hydrobiol. 134:295-51.

Vasconcelos, V.M., Sivonen, K., Evans, W.R., Carmichael, W.W., Namikoshi, M. 1996. Hepatotoxic microcystin diversity in cyanobacterial blooms collected in Portuguese freshwaters. Water Research 30:2377-2384.

.

Vasconcelos, V. 2001a. Cyanobacteria toxins: diversity and ecological effects. Limnetica 20: 45-48.

Vasconcelos, V. 2001b. Freshwater cyanobacteria and their toxins in Portugal. In: I. Chorus (Ed.) Cyanotoxins – occurrence, causes and consequences, Springer Publishers, Heidelberg, pp. 62-67.

Vasconcelos, V. 2006. Eutrophication, toxic cyanobacteria and cyanotoxins: when ecosystems cry for help. Limnetica 25: 425-432.

Vesterkvist, P.S.M., Meriluoto, J.A.O. 2003. Interaction between microcystins of different hydrophobicities and lipid monolayers. Toxicon 41: 349-355.

Wang, Q., Xie, P., Chen, J., Liang, G. 2008. Distribution of microcystins in various organs (heart, liver, intestine. Gonad, brain, kidney and lung) of wistar rat via intravenous injection. Toxicon 52: 721-727.

Weng, D., Lu, Y., Wei, Y., Liu, Y., Shen, P. 2007. The role of ROS in microcystin-LR-induced hepatocytes apoptosis and liver injury in mice. Toxicology 232: 15-23.

W.H.O. 1998. Guidelines for drinking water quality, Second Edition, Addendum to Volume 2, Health Criteria and other supporting information. World Health Organization, Geneva.

W.H.O. 2003. Cyanobacterial toxins: Microcystin-LR in Drinking-water: Background document for development of WHO *Guidelines for Drinking-water Quality*. World Health Organization, Geneva.

Wiegand, C., Pflugmacher, S. 2005. Ecotoxicological effects of selected cyanobacterial secondary metablites: a short review. Toxicol. Ppl. Pharmacol. 203: 201-218.

Woods, D., Vousden, K.H. 2001. Regulation of p53 function. Exp. Cell Res. 264, 56-66.

Wu, J.Y., Xu, Q.J., Gao, G., Shen, J.H. 2006. Evaluating genotoxicity associated with microcystin-LR and its risk to source water safety in Meiliang bay, Taihu lake. Environ. Toxicol. 21: 250-255.

Yoshida, T., Makita, Y., Nagata, S., Tsutsumi, T., Yoshida, F., Sekijima, M., Tamura, S.I., Ueno, Y. 1997. Acute oral toxicity of microcystin-LR, a cyanobacterial hepatotoxin, in mice. Natural Toxins 5: 91-95.

Yoshizawa, I., Matsushima, R., Watanabe, M.F., Harada, H., Ichihara, A., Carmichael, W.W., Fujiki, H. 1990. Inhibition of protein phosphatases by microcystis and nodularin associated with hepatotoxicity. J. Cancer Res. Clin. Oncol. 116: 609-614.

Yu, S.Z. 1995. Primary prevention of hepatocellular carcinoma, J. Gastroenterol. Hepatol. 10: 674-82.

Yuspa, S.H. 1998. The pathogenesis of squamous cell cancer: lessons learned from studies of skin carcinogenesis. J, Dermatol. Sci. 17: 1-7.

Yuspa, S.H. 2000. Overview of carcinogenesis: past, present and future. Carcinogenesis 21: 341-344.

Žegura, B., Sedmak, B., Filipič, M. 2003. Microcystin-LR induces oxidative DNA damage in human hepatoma cell line HepG2. Toxicon 41: 41-48.

Žegura, B., Lah, T., Filipič, M. 2004. The role of reactive oxygen species in microcystin-LR-induced DNA damage. Toxicology 200: 59-68.

Žegura, B., Lah, T., Filipič, M. 2006. Alteration of intracellular GSH levels and its role in microcystin-LR-induced DNA damage in human hepatoma HepG2 cells. Mut. Res. 611: 25-33.

Žegura, B., Volčič, M., Lah, T., Filipič, M. 2008a. Different sensitivities of human colon adenocarcinoma (CaCo-2), astrocytoma (IPDDC-A2) and lymphoblastoid (NCNC) cell lines to microcystin-LR induced reactive oxygen species and DNA damage. Toxicon 52: 518-525.

Žegura, B., Zajc, I., Lah, T., Filipič, M. 2008b. Patterns of microcystin-LR induced alteration of the expression of genes involved in response to DNA damage and apoptosis. Toxicon 51: 615-623.

Zhan, L., Sakamoto, H., Sakuraba, M., Wu, S., Zhang, S., Suzuki, T., Hayashi, M., Honma, M. 2004. Genotoxicity of microcystin-LR in human lymphoblastoid TK6 cells, Mut. Res. 557: 1-6.

Zhang, D., Xie, P., Liu, Y., Qiu, T. 2009. Transfer, distribution and bioaccumulation of microcystins in the aquatic food web in Lake Taihu, China, with potential risks to human health. Sci. Total Environ. 407: 2191-2199.

Zhao, Y., Xie, P., Tang, R., Zhang, X., Li, L., Li, D., 2008. *In vivo* studies on the toxic effects of microcystins on mitochondrial electron transport chain and ion regulation in liver and heart of rabbit. Comp. Biochem. Physiol., *Part C* 148, 204-210.

Zhou, L., Yu, H., Chen K. 2002. Relationship between microcystin in drinking water and colorectal cancer. Biomed Environ. Sci.15: 166-71.

Zhu, Y., Zhong, X., Zheng, S., Ge, Z., Du, Q., Zhang, S. 2005. Transformation of immortalized colorectal crypt cells by microcystin involving constitutive activation of Akt and MAPK cascade. Carcinogenesis 26: 1207-1214.

CAPÍTULO 2

Dias, E., Pereira, P., Batoréu, M.C.C., Jordan, P., Silva, M.J. 2008. Cytotoxic and genotoxic effects of microcystins in mammalian cell lines In: Moestrup, Ø. et al. (Eds), Proceedings of the 12th International Conference on Harmful Algae. ISSHA and IOC- UNESCO, Paris, pp. 282-285.

Cytotoxic and genotoxic effects of microcystins in mammalian cell lines

E. Dias[1,2], P. Pereira [2], M.C.C. Batoreu[3], P. Jordan[1] and M.J. Silva[1]
[1]Centro de Qualidade Hídrica, [2] Centro de Genética Humana, Instituto Nacional de Saúde, Av. Padre Cruz 1649-016 Lisboa, Portugal, elsa.dias@insa.min-saude.pt,
[3]Centro de Estudos de Ciências Farmacêuticas, Faculdade de Farmácia da Universidade de Lisboa, Av. Prof. Gama Pinto, 1649-043 Lisboa, Portugal

Abstract

Microcystin-LR (MCLR) has been recognized as a tumour promoter, but its carcinogenic mechanisms remain largely unknown. In this work we evaluated the genotoxic potential of microcystins (extracted from *M. aeruginosa* strains) in a mammalian cell line by the micronucleus assay (5-40 µg/mL). Cytotoxicity tests (MTT reduction, LDH release) were used to determine the sensitivity of several cell lines (Vero, HepG2 and AML12) to MCLR (1-175 µg/mL). Although all MCLR-treated cell lines presented some cytotoxic response, Vero cells were the most sensitive, showing more than 80 % decrease in viability when exposed to 22 µg/mL MCLR for 72 h. Preliminary results revealed an aneugenic or clastogenic activity of MCLR (≥ 20 µg/mL) in Vero cells. In summary, we identified a permanent mammalian cell line as a useful model system for microcystin toxicity assessment and we show that MCLR has genotoxic properties.

Introduction

The chronic effects of human exposure to low doses of microcystins (MC) are poorly understood. Microcystin-LR (MCLR) is considered a tumour promoter (Nishiwaki-Matsushima *et al.* 1992), probably through the inhibition of protein phosphatases. However, it is not clear if MCLR can also act as tumour initiator by a genotoxic mechanism. Several tests with bacteria (Grabow *et al.* 1982; Repavich *et al.* 1990; Tsuji *et al.* 1995; Ding *et al.* 1999) and human cell lines (Susuki *et al.* 1998; Zhan *et al.* 2004) have been performed to evaluate the mutagenic potential of MC. However, results are somehow contradictory and inconclusive. On the other hand, results from Comet (Rao and Battacharaya 1996; Rao *et al.* 1998; Ding *et al.* 1999; Mankiewics *et al.* 2002; Zegura *et al.* 2002, 2003; Lankoff *et al.* 2004) and Micronucleus (Ding *et al.* 1999; Zhan *et al.* 2004) assays suggest that MCs may produce DNA and chromosome breaks. Nonetheless, it remains unclear whether these effects are due to a cytotoxic or genotoxic activity and, if genotoxic, what the mechanisms are. This paper reports our first results on the analysis of cytotoxic and genotoxic effects of MC in mammalian cell lines.

Methods

1. Microcystin production from Microcystis aeruginosa cultures

Two strains of *M. aeruginosa* isolated from cyanobacterial blooms were cultured under laboratory-controlled conditions. The LMECYA7 strain is a MCLR producer (Pereira *et al.* 2001) and the LMECYA 127 is a non-toxigenic strain used to exclude eventual cyanobacterial matrix effects. Biomass from both strains was extracted with 75 % methanol and the resulting aqueous extracts were semi-purified by size exclusion followed by reverse phase preparative chromatography. The extracts were freeze dried, dissolved in the respective cell lines culture medium, and sterilized by filtration. Toxin analysis was performed for both extracts by HPLC-DAD (Watanabe *et al.* 1996).

2. Mammalian cell line maintenance

HepG2 (human hepatocellular carcinoma), AML12 (mouse hepatocytes) and Vero (African green monkey kidney) cells were obtained from the American Type Culture Collection. AML12 cells were maintained in DMEM:Ham's F12 (1:1) with 10 % foetal bovine serum (FBS), 1 % insulin-transferin-selenium mixture, and penicillin (100 U/mL)/streptomycin (100 µg/mL). HepG2 and Vero cells were maintained in MEM with 10 % FBS, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, and penicillin (100 U/mL)/streptomycin (100 µg/mL). All cells were cultured at 37 °C in a 5 % CO_2 humidified incubator. All media and supplements were purchased from Gibco BRL (Paisley, UK).

3. Cytotoxicity assays

HepG2, AML12, and Vero cells were cultured in 96-well plates (5000 cells/well) in triplicate and exposed to serial dilutions of *M. aeruginosa* extracts after cell

Moestrup, Ø. et al. (eds) Proceedings of the 12th International Conference on Harmful Algae. International Society for the Study of Harmful Algae and Intergovernmental Oceanographic Commission of UNESCO, 2008 Copenhagen

adherence. The extract from the LMECYA7 strain contained MCLR ranging from 1 to 175 µg/mL. In parallel, the non-toxigenic extract from LMECYA127 was applied at the same dilutions. Cells were exposed for 24, 48, 72, and 96 h to both extracts. The negative control consisted of cells plus culture medium. After each incubation period, LDH release to the growth medium (Legrand *et al.* 1992) and MTT reduction by adherent cells (Mossmann 1983) were determined spectrophotometrically.

4. Cytogenetic assay

The Cytokinesis-Blocked Micronucleus Assay (Fenech and Morley 1986) was used to evaluate the genotoxic potential of MCLR in Vero cells. Cultured cells were exposed for 24 h to LMECYA7 extract containing MCLR ranging from 5 to 40 µg/mL. Cytokinesis was blocked with cytochalasin B (6 µg/mL) for 24 h. Cells were fixed, spread onto glass slides, and stained according to standard protocols. For each treatment condition, 1000 binucleated cells were scored to determine the frequency of micronucleated cells.

Results

1. Cytotoxic effects

All cell lines showed a cytotoxic response when exposed to the LMECYA7 extract containing MCLR, revealed by an increase of LDH release and a decrease of MTT reduction throughout all incubation times. Fig. 1 shows the results obtained after cells were exposed for 72 h, with values corresponding to the maximum responses. In the MTT assay, Vero cells showed a 50 % decrease in viability after exposure to 11 µg/mL of MCLR. Exposure to higher doses of MCLR (22-175 µg/mL) produced an 80 % reduction in cell viability. A pronounced cytotoxicity was also observed in AML12 cells, but at a higher MCLR concentration (44 µg/mL). For HepG2 cells the viability decrease never exceeded 50 %. LDH results were inversely related to those of the MTT assay. Again, the highest cytotoxicity was obtained in Vero cells (156 % increase in LDH release at 175 µg/mL). Some variation was observed in cells exposed to the non-toxigenic extract, but they never exceeded 35 % of the control (Fig. 2). Based on these data, Vero cells were

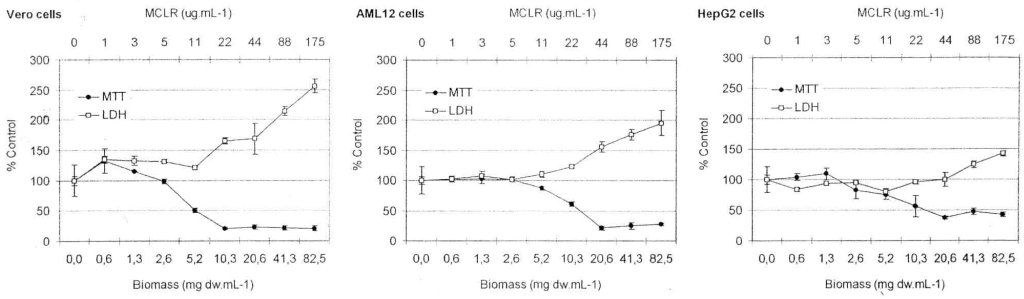


Figure 1. LDH release and MTT reduction by Vero, AML12 and HepG2 cells after exposure for 72 h to a serial dilution of LMECYA7 extract containing MCLR (1-175 µg/mL). Results are expressed as mean % ± SD of three replicates relative to the control.

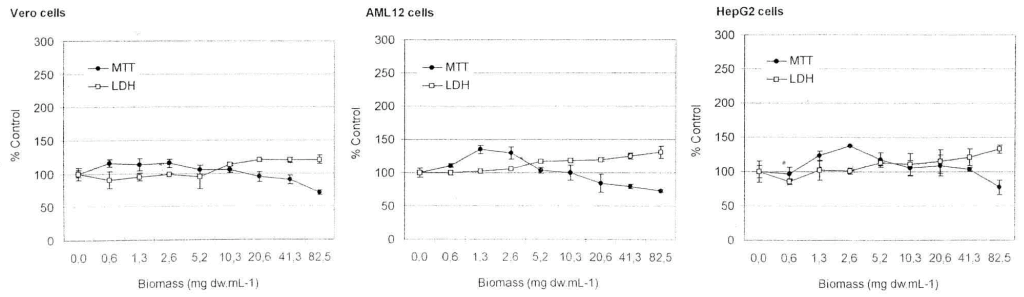


Figure 2. LDH release and MTT reduction by Vero, AML12 and HepG2 cells after exposure for 72 h to a serial dilution of non-toxigenic LMECYA127 extract. Results are expressed as mean % ± SD of three replicates relative to the control.

the most sensitive and were selected to evaluate the potential genotoxicity of MCLR.

2. Genotoxic effects

In a pilot experiment the frequency of micronuclei (MN) was analyzed in Vero cells after 24 h of exposure to a *M. aeruginosa* extract containing MCLR (Fig. 3). MCLR concentrations of 20 and 40 µg/mL increased 3.4- and 4.1-fold the number of cells with MN, respectively, compared to the control. A linear dose-response relationship between the frequency of micronucleated cells and the MCLR concentration was obtained (t test for a Pearson correl. coeff., p = 2,81E-4).

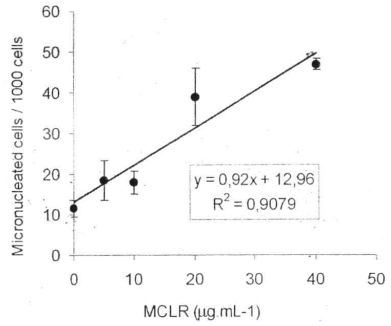


Figure 3. Induction of Micronuclei in Vero cells exposed to a *M. aeruginosa* extract (LMECYA7) containing 5, 10, 20 and 40 µg/mL of MCLR for 24 h. Data are expressed as mean ± SD of two replicates.

Discussion

The organotropism of MC has been attributed to the mechanism of their uptake by cells. MC enters the cells through the Organic Anion Transporting Polypeptides (OATP) (Fisher *et al.* 2005), present mainly in the liver and to a much lesser extent in other organs. It has been assumed that primary hepatocytes preserve these transporters, but that permanent cell lines tend to lose them. This assumption and the failure of some authors to obtain toxic responses in mammalian cell lines in vitro may explain, at least in part, the relatively low number of toxicological studies on microcystins using permanent cell lines as the experimental model.

In this work we showed that *M. aeruginosa* extracts containing MCLR induced strong cytotoxic responses in human (HepG2), monkey (Vero), and mouse (AML12) cell lines. The failure of a non-toxigenic *M. aeruginosa* extract to cause a similar effect lead us to conclude that microcystin-LR was responsible for the cytotoxicity. Interestingly, and contrary to what we expected, the most sensitive cells were the non-liver derived Vero cells. No previous data have been published regarding the cytotoxic effects of microcystins in AML12 cells. However, Boaru *et al.* (2006) and Chong *et al.* (2000) reported that HepG2 and Vero cells are insensitive to MCLR. These contradictions might be explained by the use of different experimental set-ups. Those authors used pure toxin, whereas we used semi-purified toxin. We did not detect other microcystins besides MCLR in the LMECYA7 extract. Thus, our results can not be justified by a synergistic effect of different toxin variants. However, the complex and unidentified cyanobacterial matrix may somehow influence MCLR toxicity. The present work points to the usefulness of Vero cells as a model to study microcystin toxicity and suggests that this permanent cell line might preserve their OATP, or that MCLR uptake/toxicity might be triggered by another unknown mechanism. On the other hand, the observed induction of micronuclei in Vero cells by the toxic *M. aeruginosa* extract suggests that MCLR has a genotoxic activity. This raises the question of whether MCLR acts as a clastogenic or an aneugenic agent and supports the previous suggestion that MC can act as tumour initiators. Further studies using centromere labelling by fluorescence *in situ* hybridization will clarify the mechanism behind the genotoxic effect.

Acknowledgements

This work was funded by the Fundação para a Ciência e Tecnologia, Portugal (PhD grant to E. Dias SFRH/BD10585/2002 and Project POCI/AMB/60351/2004).

References

Boaru, D.A., Dragos, N. & Schirman, K. (2006). Toxicology 218: 134-148.

Chong, M., Gu, K., Lam, P., Yang, M. & Fong, W. (2000). Chemosphere 41: 143-147.

Fenech, M. & Morley, A.A. (1986). Mut. Res. 161: 193-198.

Fischer, W.L, Altheimer, S., Cattori, V., Meier, P.J., Dietrich, D.R. & Hagenbuch, B. (2005). Toxicol. Appl. Pharmacol. 203: 257-263.

Grabow, W., Randt, W., Prozesky, O. & Scott, W. (1982). Appl. Environ. Microbiol. 43: 1425-1433.

Lankoff, A., Krzowski, L., Glab, J., Banasik, A., Lisowska, H., Kuszwski, T., Gozdz, S. & Wojcik, A. (2004). Mut. Res. 559: 131-142.

Legrand, C., Bour, J.M., Jacob, C, Capiaumont, J., Martial, A., Marc, A., Wudtke, M., Kretzmer, G.,

Demangel, C. & Duval, D. (1992). J. Biotechnol.. 25: 231-243.

Mankiewicz, J., Walter, Z., Tarczynska, M., Palyvoda, O., Wojtysiak, S.M. & Zalewski, M. (2002). Environ. Toxicol. 17: 341-350.

Mossmann, T. (1983). J. Immunol. Meth. 65: 53-63.

Nishiwaki-Matsushima, R., Ohta, T., Nishiwaki, S., Suganuma, M., Ishikawa, T., Carmichael, W.W. & Fujiki, H. (1992). J. Cancer Res. Clin. Oncol. 118: 420-424.

Pereira, P., Onodera, H., Andrinolo, D., Franca, S., Araújo, F., Lagos, N. & Oshima, Y. (2001). In: Harmful Algal Blooms 2000, Hallegraeff, G.M., Blackburn, S.I., Bolch C.J., and Lewis, R.J. (eds), UNESCO, Paris, pp. 108-111.

Rao, P.V. & Bhattacharya, R. (1996). Toxicology 114: 29-36.

Rao, P. V., Bhattacharya, R, Parida, M.M., Jana, A.M. & Bhaskar, A.S. B. (1998). Environ. Toxicol. Pharmacol. 5: 1-6.

Repavich, W.N., Sonzongni, J.H., Standridge, R.E., Wedphohl, L.F. & Meisner, L.F. (1990). Water Res. 24: 225-231.

Suzuki, H., Watanabe, M.F., Wu, Y., Sugita, T., Kita, K., Sato ,T., Wang, X., Tanzawa, H., Sekiya, S. & Suzuki, N. (1998). Int. J. Mol. Med. 2: 109-112.

Tsuji, K., Watanuki, T., Kondo, F., Watanabe, M.F., Suzuki, S., Nakazawa, H., Suzuki, M., Uchida, H. & Harada, K. (1995). Toxicon 33: 1619-1631.

Watanabe, M., Harada, K., Carmichael, W.W. & Fujiki, H. (1996). Toxic *Microcystis*. CRC Press, New York.

Zhang, Z., Yu, S. & Chen, C. (2001). Zhonghua Yu Fang Yi Xue Za Zhi 35: 75-78.

Zhan, L., Sakamoto, H., Sakuraba, M., Wu, D.S., Zhang, L. S, Suzuki, T., Hayashi, M. & Honma, M. (2004). Mut. Res. 557: 1-6.

Zegura, B., Sedmak, B. & Filipic, M. (2003). Toxicon 41: 41-48.

Zegura, B., Filipic, M., Suput, D., Lah, T. & Sedmak, B. (2002). Radiol. Oncol. 36: 159-161.

CAPÍTULO 3

Dias, E., Andrade, M., Alverca, E., Pereira, P., Batoréu, M.C.C., Jordan, P., Silva, M.J. 2009. Comparative study of the cytotoxic effect of microcistin-LR and purified extracts from Microcystis aeruginosa on a kidney cell line. Toxicon 53, 487-495.

Toxicon 53 (2009) 487–495

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Comparative study of the cytotoxic effect of microcistin-LR and purified extracts from *Microcystis aeruginosa* on a kidney cell line

Elsa Dias [a,b,c,*], Mariana Andrade [b], Elsa Alverca [b], Paulo Pereira [b], M.C.C. Batoréu [c], Peter Jordan [a], Maria João Silva [a]

[a] National Institute of Health Dr. Ricardo Jorge, Department of Genetics, Av. Padre Cruz, 1649-016 Lisbon, Portugal
[b] National Institute of Health Dr. Ricardo Jorge, Department of Environmental Health, Av. Padre Cruz, 1649-016 Lisbon, Portugal
[c] University of Lisbon, Faculty of Pharmacy, I-Med, Laboratory of Toxicology, Av. Prof. Gama Pinto, 1649-019 Lisbon, Portugal

ARTICLE INFO

Article history:
Received 26 November 2008
Received in revised form 27 January 2009
Accepted 28 January 2009
Available online 5 February 2009

Keywords:
Microcystin-LR
Cytotoxicity
Vero cells
Kidney

ABSTRACT

Microcystin-LR (MCLR) is a potent hepatotoxin, but increasing evidences suggest that it might also induce kidney injury. The aim of this work was to evaluate the cytotoxicity of MCLR on a kidney cell line (Vero-E6). Cells were exposed for up to 72 h either to *Microcystis aeruginosa* extracts from both MCLR-producer and non-MCLR-producer isolates or to pure MCLR (1.5–200 μM). The cytotoxic effects were evaluated by several cell viability assays (MTT, Neutral Red and LDH). Pure MCLR, the extract from MCLR-producer and the mixture of the non-MCLR-producer with pure MCLR, induced cell viability decrease in a similar dose/time-dependent manner. Conversely, no effects were induced by the extract of non-MCLR-producer. These results suggest that the cytotoxic effects of *M. aeruginosa* extract were due to MCLR and excluded the eventual toxicity of other cyanobacteria bioactive compounds. The lowest cytotoxic MCLR concentration varied between 11 and 100 μM depending on the employed cell viability assay and is within the range of MCLR dosage reported to affect other mammalian cell lines. The NR assay was the most sensitive to evaluate the MCLR-induced cytotoxicity. Our results suggest that Vero-E6 cell line may constitute a cell model to evaluate the nephrotoxicity of microcystins.

1. Introduction

Microcystins are a family of hepatotoxic heptapeptides produced by freshwater cyanobacteria. The knowledge of their severe hepatotoxicity has been derived both from acute human and animal poisoning incidents (Duy et al., 2000; Chorus et al., 2000) and from *in vivo* and *in vitro* experimental studies (Runnegar and Falconer, 1982; Aune and Berg, 1986; Eriksson et al., 1989; Mereish and Solow, 1990; Yoshizawa et al., 1990; Gupta et al., 2003). Microcystins act by inhibiting serine/threonine protein phosphatases 1 and 2A in the liver cells (Yoshizawa et al., 1990). This induces overphosphorylation of cytoskeletal filaments, leading to the collapse of liver tissue organization, liver necrosis and, eventually, acute intrahepatic bleeding (Falconer and Yeung, 1992). The chronic ingestion of small doses has also been associated to human primary hepatocellular carcinoma (Ueno et al., 1996). In 1998, the World Health Organization defined a drinking water guideline value of 1.0 $\mu g\,L^{-1}$ for microcystin-LR (MCLR), the most toxic and widespread microcystin variant, revealing the importance of these toxins as a potential public health hazard (WHO, 1998).

The organotropism of microcystins has been attributed to a specific transport system – the Organic Anion Polypeptide Transporter (OAPT) – in the hepatocytes that mediates the uptake of a wide spectrum of amphipathic organic solutes into the cells. Some of the members of this transporter superfamily are selectively expressed in the

* Corresponding author at: National Institute of Health Dr. Ricardo Jorge, Department of Environmental Health, Av. Padre Cruz, 1649-016 Lisbon, Portugal. Tel.: +351 217519393; fax: +351 217526400.
E-mail address: elsa.dias@insa.min-saude.pt (E. Dias).

0041-0101/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.toxicon.2009.01.029

liver and are involved in the uptake and elimination of numerous xenobiotics, including microcystins (Hagenbuch and Meier, 2003). This system is absent or present at a lower extent in cells from other organs and is lost by hepatocytes during *in vitro* culture procedures for the set up of permanent cell lines (Runnegar et al., 1991; Fischer et al., 2005; Boaru et al., 2006).

Though microcystins are primarily hepatotoxic, some papers have already described an *in vivo* nephrotoxic activity of MCLR in rats (Nobre et al., 1999; Multinović et al., 2002, 2003). Additionally, and despite the apparent insensitivity of permanent cell lines to microcystins, Khan et al. (1995) demonstrated that MCLR was able to induce morphological and ultrastructural effects on a kidney cell line. Moreover, one of the known microcystin's carriers (OATP-A) has been recently identified at mRNA level in the human kidney (Hagenbuch and Meier, 2003). These data suggest that the kidney might also be an important target organ for microcystins and that the risk of kidney damage resulting from the human exposure to these toxins should be more accurately characterized.

The objectives of this work were to compare the cytotoxic effect of pure MCLR and of MCLR from *Microcystis aeruginosa* extracts in a monkey kidney cell line, Vero-E6. Using several cytotoxicity assays we show that Vero-E6 cell line is a useful cell model to study the nephrotoxicity of microcystins. In these cells, MCLR triggered a dose- and time-dependent cytotoxic response, irrespective of the origin of the toxin: commercial or prepared from an *M. aeruginosa* strain isolated from a natural bloom. Our data also show that the neutral red assay is the most sensitive method to evaluate the MCLR toxicity in Vero cells, as compared to MTT and LDH cell viability assays.

2. Materials and methods

2.1. Microcystin-LR and cyanobacteria extracts

Microcystin-LR was purchased from Sigma–Aldrich (CAS Number 101043-37-2) as a white solid film (purity $\geq$95%, by HPLC). A stock solution of MCLR (1 mM) was prepared by dissolving the toxin in cell culture medium. This solution was sterilized by filtration through 0.22 μm filters (Millex-GV, PVDF, Millipore) and kept at −20 °C until use. This form of MCLR is thereafter named "pure MCLR".

The cyanobacteria extracts were prepared from two *M. aeruginosa* strains (LMECYA 7 and LMECYA 127) isolated in 1996 from Montargil reservoir (Portugal) and successfully maintained in the laboratory as monoalgal, free of eukaryotes, non-axenic cultures. Toxin analysis of LMECYA 7 was characterized in a previous report (Pereira et al., 2001) showing that this strain produces exclusively the MCLR variant of microcystins. The determination of microcystins by HPLC-DAD in the LMECYA 7 extract used here as working solution also confirmed that the process of extract preparation did not influence the microcystin composition of the LMECYA 7. The LMECYA 127 was used for control purposes as a non-microcystin producer. Cultures of both isolates were grown in plankton light reactors (Aqua-Medic, Bissendorf, Germany) containing 2.5 L of Z8 medium (Skulberg and Skulberg, 1990) under continuous aeration, in a 16/8 h L/D cycle (light intensity 30 $\mu E\ m^{-2}\ s^{-1}$, aprox.) at 22 ± 1 °C. Cells harvested during late 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 under magnetic stirring. The cell suspensions were further sonicated with an ultrasonic probe (Sonics Vibra-Cell CV33, Sonics & Materials Inc., CA, USA). The extracts were centrifuged and the pellets were re-extracted by the same procedure. Supernatants of the two extractions were combined and subjected to rotary evaporation to eliminate methanol. The resulting aqueous extracts were subjected to solid phase extraction for microcystin clean-up on Sep-PakC18 cartridges (500 mg, Millipore, Bedford, MA, USA). The cartridges were previously activated with 10 mL of methanol (100%) and 10 mL of water. After applying the extract, the cartridge was rinsed with 10 mL of water and 5 mL of methanol 20%. Microcystins were then eluted with 20 mL of 80% methanol and collected in glass vials. The eluted fraction was evaporated to dryness in a Speed-Vac system (AES 1000, Savant) and re-suspended in cell culture medium. The final extracts were sterilized by filtration through 0.22 μm filters (Millex-GV, PVDF) and kept at −20 °C until use. The extracts were analysed by HPLC-DAD for microcystin's quantification using a Shimadzu LC10A system (Shimadzu, Kyoto, Japan) following the method of Harada (1996).

2.2. Vero cell line maintenance

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 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%, Invitrogen), centrifuged (300 × *g*) and the cell viability was determined by the trypan blue dye exclusion method (Philips, 1973). 5000 viable cells were seeded per individual 96-microplate wells and cultured for 24 h for cell adherence and growth.

2.3. Cell exposure to M. aeruginosa extracts and pure MCLR

After the 24 h incubation period, the growth medium was replaced by serial dilution of the LMECYA 7 extract in fresh growth medium, corresponding to final concentrations of MCLR from 1.4 up to 175 μM. The same biomass dilutions (from 0.6 to 83 $mg\ mL^{-1}$) of LMECYA127 extract (devoid of microcystins) were tested in parallel to evaluate the eventual toxicity of *M. aeruginosa* extract matrix. The negative control consisted of cells grown in fresh culture medium. For both extracts the cells were exposed for 24, 48 and 72 h and the cytotoxic effect was evaluated by the 3-(4,5-Dimethyl-2-thizaolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and lactate dehydrogenase (LDH) assays.

To further evaluate the influence of the matrix of *M. aeruginosa* extracts on microcystin's toxicity, Vero

cells were exposed for 24 h to serial dilutions of the MCLR stock solution (from 1.5 to 100 μM) and, in parallel, to the LMECYA127 extract spiked with pure MCLR at equivalent concentrations. The cell viability was assessed by the MTT assay.

A similar procedure was followed to test the cytotoxic effects of pure MCLR. Vero cells were exposed to serial dilutions of MCLR stock solution in fresh growth medium, corresponding to final concentrations of 1.5–200 μM of MCLR. In a first trial, the cytotoxicity was evaluated by the neutral red (NR), MTT and LDH assays after 24, 48 and 72 h of exposure. In a second trial, the cytotoxicity was assessed by the NR assay after 2, 6 and 12 h of exposure.

All the exposure conditions were tested in triplicate for cytotoxicity and photomicrographs were taken for cell morphological analysis, using an Olympus CK40 inverted microscope coupled with an Olympus PM-20 camera.

2.4. Cytotoxicity evaluation by LDH, MTT and NR assays

The lactate dehydrogenase leakage into extracellular medium reflects the loss of cell viability due to cell membrane damage. After each incubation period, the exposure medium was transferred to a new microplate and centrifuged at $250 \times g$ for 4 min to remove cells in suspension and cell debris. LDH activity was determined in the clean supernatant (Legrand et al., 1992) using the commercial Tox-7 kit (Sigma, St. Louis, MO, USA) according to the manufacturer procedure.

The MTT assay measures the reduction of the 3-(4,5-Dimethyl-2-thizaolyl)-2,5-diphenyl-2H-tetrazolium bromide into an insoluble and impermeable compound (formazan) by mitochondria dehydrogenases, which accumulate in healthy cells. This assay was performed with the adherent cells according to the method of Mossmann (1983). After the exposure period, the medium was removed and the cells were incubated for 3 h at 37 °C with fresh growth medium containing 10% of MTT solution (5 mg mL^{-1} in PBS, Calbiochem, Darmstadt, Germany). To dissolve the MTT–formazan crystals, the MTT containing medium was discharged and a solution of acidified propan-2-ol solution (0.04 M HCl) was added for 15 min under shaking. The absorbance was recorded at 570 nm using a Multiscan Ascent spectrophotometer (Labsystems, Helsinki, Finland).

The Neutral Red assay measures the incorporation of the NR dye by the lysosomes of viable cells (Borenfreund and Puerner, 1985). At each time point, the exposure medium was replaced by fresh growth medium containing 10% of NR solution (50 μg mL^{-1}, Merck, Darmstadt, Germany). After a 3 h incubation period, cells were rinsed with PBS and the incorporated NR was extracted with a mixture of ethanol:acetic acid:water (50:1:49). NR incorporation was quantified spectrophotometrically at 540 nm.

2.5. Data and statistical analyses

Results are presented as mean % ± standard deviation of three replicates relative to the control values. Data from LDH assay were converted to 1/ABS rather than absorbance itself, in order to be graphically compared to data from the MTT and NR assays. An arbitrary threshold of 50% cell viability was considered as an indicator of a marked cytotoxic effect. Statistical differences were analysed with a Factorial ANOVA followed by the Tukey's test. Values of $p < 0.05$ were considered as statistically significant.

3. Results

The effects of MCLR-producer LMECYA7 and non-MCLR-producer LMECYA 127 cyanobacteria extracts on Vero cells, evaluated by MTT and LDH assays, are shown in Fig. 1. The viability of cells exposed to LMECYA 7 decreased significantly ($p < 0.001$) with MCLR concentrations and time of exposure in both assays. The lowest toxin concentration needed to induce a significant ($p < 0.05$) and marked cytotoxic response (cell viability ≤ 50%) by the MTT assay was 22 μM at 24 h and 11 μM at 48 h and 72 h. By the LDH assay, although a significant decrease in cell viability was observed above 22 μM of MCLR at 72 h, a marked cytotoxic effect was only detected after exposure to the highest toxin concentration (175 μM). On the other hand, no cytotoxic effects were detected in cells exposed to several dilutions and time points of the extract from the non-MCLR-producer cyanobacteria LMECYA 127. In fact, no differences in cell viability measured by MTT ($p > 0.2$) and LDH ($p > 0.1$) assays were found between the cells treated with this extract and the negative control (Fig. 1).

Cells exposed to the extract from the non-MCLR producer spiked with pure MCLR showed values of cell viability similar to those exhibited by cells exposed to equivalent concentrations of pure MCLR by the MTT assay (Fig. 2). For both treatments, it can be seen that cell viability decreases significantly with increasing MCLR concentration ($p < 0.0001$), and no significant differences were found between the two dose–response curves ($p > 0.9$).

Fig. 3 shows the cytotoxic effects of pure MCLR on Vero cells treated for 24 h, 48 h and 72 h and evaluated by NR, MTT and LDH assays. Concerning the MTT and LDH assays, the results show a pattern similar to those obtained with LMECYA 7 extract. Both assays showed a significant ($p < 0.0001$) dose- and time-dependent cytotoxic effect of MCLR on cell viability. The lowest MCLR concentration required to elicit a significant cytotoxic response by the MTT assay at 24 h (25 μM) was quite similar to that of LMECYA 7 extract (22 μM) but a more pronounced decrease in cell viability occurred for higher pure toxin concentrations. However, for the 48 and 72 h treatments, lower MCLR concentrations in the LMECYA 7 extract were needed to achieve a significant difference over control and higher cytotoxicity levels. On the other hand, as observed for the toxic LMECYA 7 extract, the effects of pure MCLR evaluated by the LDH assay only became significant at toxin concentrations higher than those shown to be cytotoxic by the MTT assay (200 μM at 24 and 48 h; 100 μM at 72 h).

Data from the NR assay also show that pure MCLR induces a significant dose- and time-dependent decrease in Vero cells viability ($p < 0.0001$). The lowest MCLR concentrations that induced marked cytotoxicity were 50 μM for the 24 h treated cells and 25 μM for the 48 h and 72 h treatments. However, the decrease of cell viability determined by the NR assay was, in general, more pronounced than that detected by the MTT assay. This was particularly

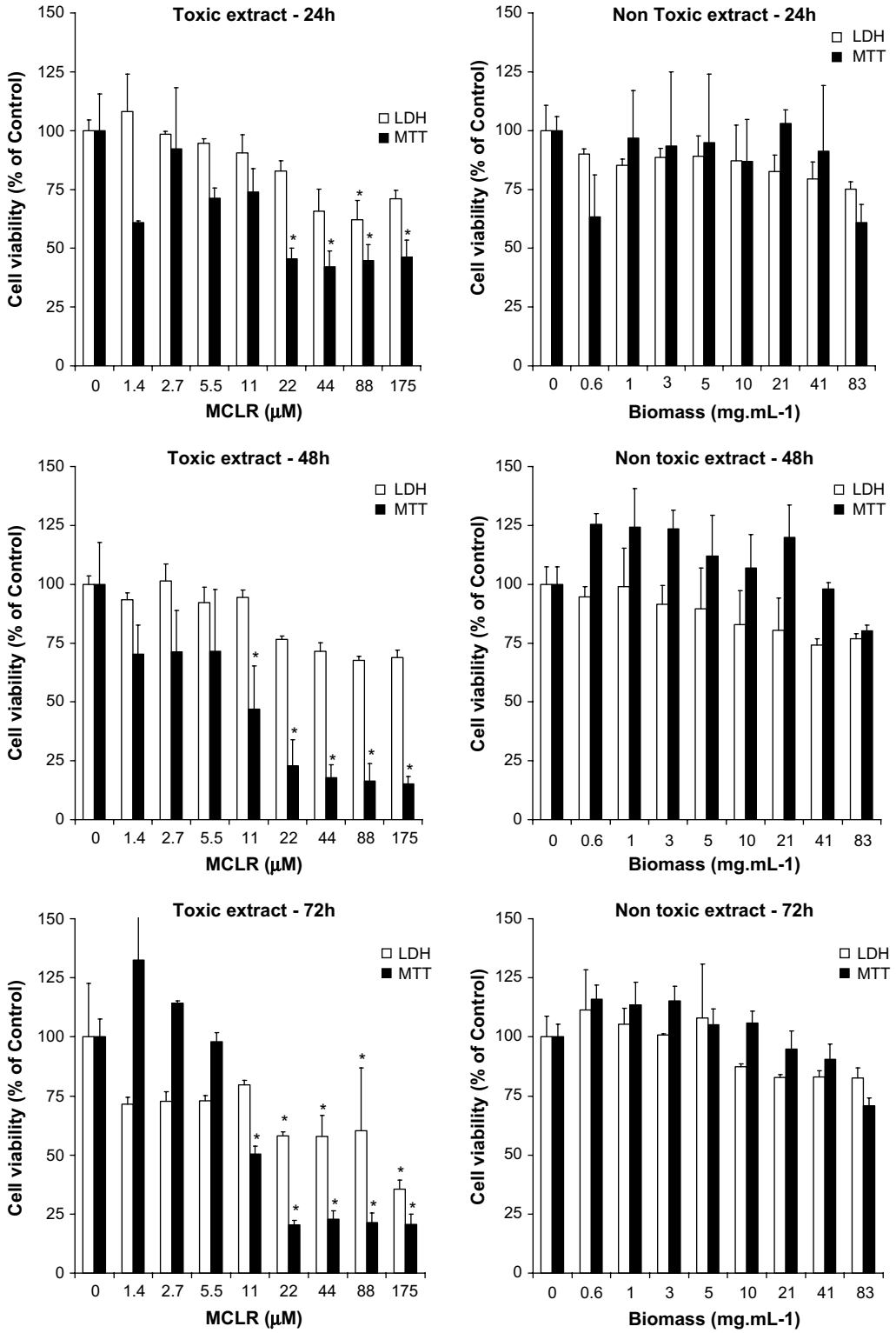


Fig. 1. Viability of Vero-E6 cell line exposed for 24, 48 and 72 h to a toxic *M. aeruginosa* extract containing 1.4–175 μM of MCLR (on the left) and to a non-MCLR-producer *M. aeruginosa* extract (on the right). The two extracts were applied at the same biomass dilutions. Cell viability was assessed by the MTT and LDH assays. Results are expressed as the mean percentage of three replicates relative to control ± standard deviation. * represents a statistically significant difference between the treated and the control cells ($p < 0.05$).

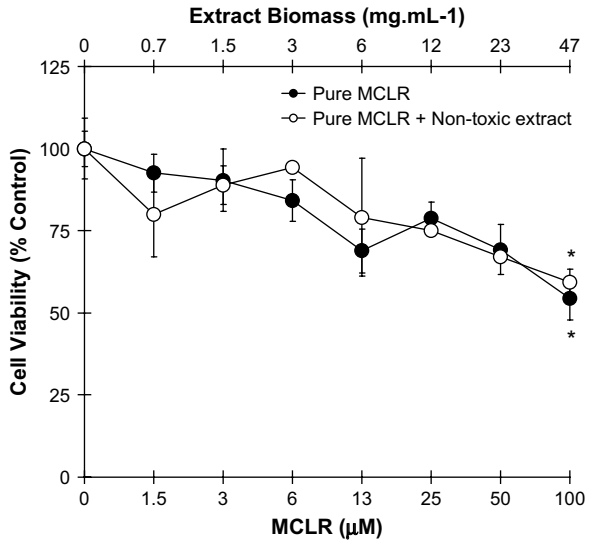


Fig. 2. Comparison of the cytotoxicity induced by pure MCLR (1.5–100 μM) and by a mixture of the non-MCLR-producer *M. aeruginosa* extract (0.7–47 mg dw mL^{-1}) spiked with MCLR (at equivalent concentrations) on Vero-E6 cell line. Cytotoxicity was evaluated by the MTT assay after 24 h of exposure of Vero cells to both solutions. The secondary *x*-axis represents the corresponding biomass concentrations (mg dw mL^{-1}). Results are expressed as the mean percentage of three replicates relative to control ± standard deviation. * represents a statistically significant difference between the treated and the control cells ($p < 0.05$).

evident for the 48 h and 72 h exposure experiments where higher MCLR doses were needed for MTT assay to detect cell viabilities losses similar to the ones detected by the NR assay.

Given this apparently higher sensitivity of NR assay to detect the cytotoxic effects of MCLR, we searched for MCLR-induced cytotoxicity at lower exposure periods (Fig. 4). After 12 h of exposure, a significant decrease in cell viability was detected above 25 μM of MCLR in comparison to control, but the loss of cell viability never exceeds 55%. No significant effects were detected with shorter exposures (2 h and 6 h), even in cells treated with high toxin doses (100 and 200 μM).

Fig. 5 shows optical photomicrographs of Vero cells exposed to pure MCLR and to each of the *M. aeruginosa* extracts. Control cells and cells exposed to the non-toxic LMECYA 127 extract displayed a similar high confluence and typical polyhedral morphology (Fig. 5A and B). Cultures exposed to the LMECYA 127 extract spiked with pure MCLR (100 μM) presented a lower cell confluence and a high proportion of rounded cells i.e., cells detached from the culture monolayer (Fig. 5C). Similar effects on the cell morphology were observed after treatment with equimolar doses of pure MCLR and toxic LMECYA 7 extract (Fig. 5D and E). For both treatments, the effects of MCLR on cell morphology were dose- and time-dependent, being detectable within the first 24 h of exposure to 20 μM of MCLR (data not shown) and more pronounced at 50–100 μM (Fig. 5C–E). With MCLR concentrations above 100 μM, the majority of the cells rounded up and detached from the dish (Fig. 5F). These effects were also detected at

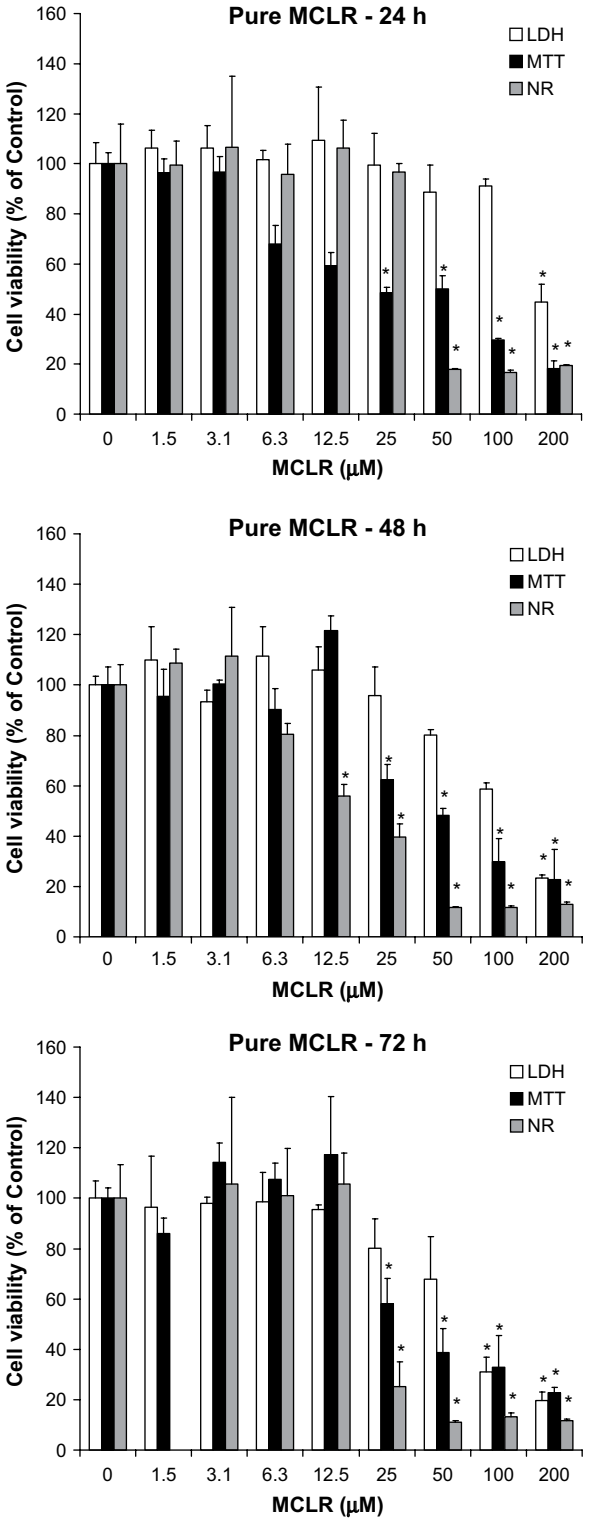


Fig. 3. Viability of Vero-E6 cell line exposed to pure MCLR (1.5–200 μM) for 24, 48 and 72 h, assessed by NR, MTT and LDH assays. Results are expressed as the mean percentage of three replicates relative to control ± standard deviation. * represents a statistically significant difference between the treated and the control cells ($p < 0.05$).

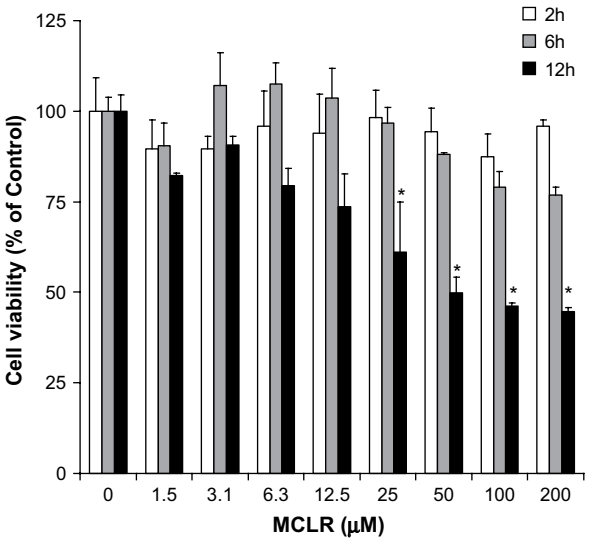


Fig. 4. Viability of Vero-E6 cell line exposed to pure MCLR (1.5–200 μM) for 2, 6 and 12 h, assessed by NR assay. Results are expressed as the mean percentage of three replicates relative to control ± standard deviation. * represents a statistically significant difference between the treated and the control cells ($p < 0.05$).

lower toxin concentrations for longer incubation periods (Fig. 5G).

4. Discussion

The toxicity of microcystins is relatively well characterized in hepatic cell models, given the fact that liver is the target organ for these environmental toxins. However, increasing evidence suggests that microcystins might also induce toxic effects on other organs such as the kidney.

In a previous work, we reported the cytotoxic effects of *M. aeruginosa* extracts in human (HepG2), monkey (Vero) and mice (AML12) cell lines (Dias et al., 2008). Interestingly, when exposed to extracts containing equivalent microcystins concentrations, kidney-derived Vero cells showed to be more strongly affected than the liver-derived HepG2 and AML12 cell lines. However, the use of cyanobacteria extracts to evaluate toxicological properties of microcystins requires careful validation. It is well known from the literature that crude or semi-purified extracts of *Microcystis* are likely to contain bioactive compounds other than microcystins which might be responsible for, or contribute to, the observed toxicity (Falconer, 2007). Therefore, the observed effects could not be unequivocally attributed to microcystins prior to checking if the pure toxin actually had the same effect.

In this work, we demonstrate that the cytotoxic effects of MCLR-containing cyanobacteria (LMECYA 7) extract on Vero cells are comparable to those induced by commercially available MCLR (pure MCLR). Cells exposed to several extract dilutions showed cell viability decrease and morphological defects similar to those treated with equivalent doses of pure MCLR. In addition, MCLR produced similar cytotoxic effects either applied in a pure state or mixed with the non-toxic cyanobacteria extract. Such effects were not detected in cells exposed to the extract from a non-MCLR producer. Thus, the complex matrix of cyanobacteria extracts showed no activity, either synergistic or antagonistic, on MCLR toxicity. Our data disagree with some previous studies that have shown that crude cyanobacterial extracts may result in stronger toxic effects than pure microcystins when applied in equivalent concentrations (Falconer, 2007; Pietsch et al., 2001). A possible explanation for this discrepancy is that our assays were performed with extracts previously subjected to a clean-up procedure that might have eliminated eventual bioactive substances from the crude cyanobacteria extracts.

To our knowledge, there are only few reports regarding the effects of microcystins on Vero cell line. Thompson et al. (1987) found no effects on the morphology and on LDH release of Vero cells treated with cyanobacteria extracts containing up to 10 μM of MCLR. Grabow et al. (1982) reported cytophatogenic effects (rounding and disintegration of cells) caused by *M. aeruginosa* extracts containing 500 μM of MCLR. In a more recent report, Chong et al. (2000) evaluated the sensitivity of Vero cells to pure MCLR by the MTT assay and found no effects with toxin concentrations of up to 37.5 μM for 24–96 h treatments. The data from these reports can hardly be comparable given that the authors used toxins from different sources (pure or crude extracts), applied them in different dosages and evaluated the effects by different end points. In our experiments, clear morphological signs of cell injury were observed at 25–50 μM MCLR/24 h exposure and MTT results showed a significant decrease in cell viability at 25 μM MCLR/24 h. These results show a higher sensitivity of Vero cells to MCLR than that previously reported. The differences might result in part from the use of different clones of Vero cells. The cell line used in our experiments was from kidney epithelial cells derived from the African green monkey (ATCC-CRL 1586). It is possible that Grabow et al. (1982) and Chong et al. (2000) have used different Vero cell lines, showing different sensitivities to MCLR.

The cytotoxic effects on Vero cells detected in our work were induced within the MCLR dose-range reported to affect the viability of other permanent cell lines. Khan et al. (1995) found MCLR to exert morphological changes on a rat kidney epithelial cell line (NRK-52E) at 100 μM MCLR/24 h, while McDermott et al. (1998) reported the onset of cytotoxic effects in different human permanent non-liver cell lines at similar MCLR dose/time treatments. Using Caco-2 (human colon carcinoma) and MCF-7 (human breast adenocarcinoma) cells, Botha et al. (2004) detected a decrease in the cell viability by both MTT and LDH assays at 50 μM of MCLR/24 h. Chong et al. (2000) reported cytotoxic effects by MTT test on KB (oral epidemoid carcinoma), NIH (mouse embryo) and H-4-II-3 (rat hepathoma) cell lines for lower MCLR doses, but for longer exposure periods (18.75 μM MCLR/96 h). Our results from the MTT assay showed that the viability of Vero cells was significantly affected by MCLR, at doses similar or slightly lower than those reported in most of these studies. However, higher MCLR concentrations were needed to obtain significant detectable effects by the LDH method (200 μM MCLR/24 h and 100 μM/72 h). On the other hand, the mean values of cell viability measured by the NR assay were, in general,

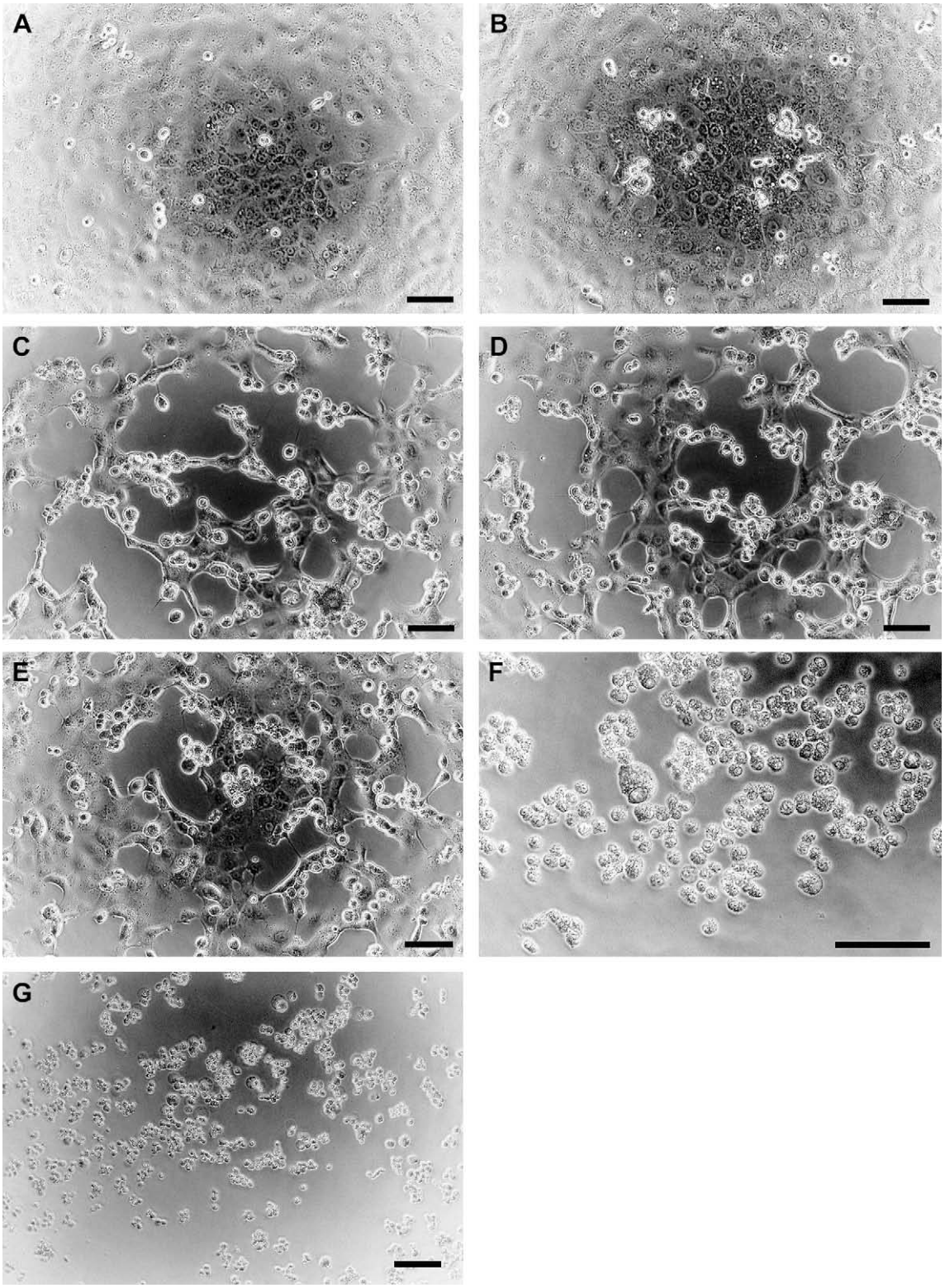


Fig. 5. Morphology of Vero cells exposed for 24 h to (A) culture medium (negative control); (B) non-toxic (NT) *M. aeruginosa* extract; (C) NT extract spiked with MCLR (100 μM); (D) pure MCLR (100 μM); (E) toxic *M. aeruginosa* extract (100 μM MCLR); (F) toxic *M. aeruginosa* extract (175 μM MCLR); (G) toxic *M. aeruginosa* extract (44 μM MCLR/48 hours). Scale bars = 100 μm.

lower than the ones measured by MTT at equivalent MCLR treatments. This suggests that NR assay is the most suitable to evaluate MCLR-induced cytotoxicity.

The differences obtained from each assay might reflect a differential mode of action of MCLR in the cells. While the NR assay reproduces the lysosome integrity, the MTT assay relates to the mitochondrial activity and the LDH assay to cell membrane damages. Thus, the higher sensitivity revealed by the NR assay, especially at longer exposure periods, suggests that the lysosomes are primarily affected by MCLR and then the mitochondria and the cell membrane. This apparently higher sensitivity of lysosomes to MCLR, has also been reported for other cells. While testing the effects of MCLR on primary fish hepatocytes, Boaru et al. (2006) found that the toxin dose required to attain a significant effect on lysosomes (0.25 μM, NR assay) was lower than that needed to affect similarly the mitochondria and the endoplasmatic reticulum (2.5 μM, Alamar Blue assay). The authors also evaluated the disruption of the plasma membrane by the CFDA–AM assay and did not find any effect caused by MCLR, even at higher toxin dose (2.5 μM). Using fish cell lines, Pichardo et al. (2005) reported that significant effects were detected by NR assay with 10 μM of MCLR while no LDH release was determined up to 150 μM. Thus, our data on Vero cells support the indication that the NR assay is an important tool to evaluate cytotoxic effects of MCLR in different permanent cell lines. When applied simultaneously with other end point assays, it might also give important insights on the toxic action of MCLR at a sub-cellular level. Indeed, further studies conducted in our laboratory (data not published) revealed that purified extracts from toxic *M. aeruginosa* affected several cellular organelles of Vero cells and, in particular, the lysosomes.

MCLR concentrations required to trigger a cytotoxic response on Vero cells (11–25 μM) are within the range of previously reported levels of microcystins found in natural environments (Sivonen and Jones, 1999; Funari and Testai, 2008). Such levels are usually found in cyanobacterial blooms reaching extremely high cell densities.

Several comparative studies have shown that permanent cell lines require more prolonged exposures and higher MCLR doses to develop the same cytotoxic response than primary hepatic cells (Khan et al., 1995; McDermott et al., 1998; Boaru et al., 2006). This has been attributed to the lack/decrease of OATP expression by continuous cell cultures (Boyer et al., 1993; Boaru et al., 2006). We observed that MCLR concentrations required to trigger a toxic response in Vero cells were similar to those reported for other mammalian cell lines. However, those concentrations were 10–1000 times higher than those previously reported to induce cytotoxicity in primary hepatocytes. To our knowledge, the expression of OATP in Vero cells has not yet been described. However, at least one microcystin carrier (OATP-A) has already been identified at mRNA level in the human kidney (Hagenbuch and Meier, 2003). Further research is needed in order to clarify whether differences in OATP expression might explain different sensitivities of non-liver cells to microcystins.

Despite the high toxin levels needed to induce cytotoxic effects on Vero cells, we consider that this cell line might constitute a valuable model to study the toxicity of microcystins. In fact this high threshold should not drive us to conclude that MCLR is not able to exert a toxic effect on kidney at lower doses. Nobre et al. (1999) reported renal vascular, glomerular and urinary effects of MCLR on rat kidneys perfused with 1 μM toxin solution for 120 min. Milutinović et al. (2002) also showed that chronic treatment of rats with intraperitonial injections of sublethal doses of microcystins (10 $\mu g\,Kg^{-1}$) could induce severe kidney injuries. The authors further showed that the mechanisms that underlie the chronic nephrotoxicity of microcystins are similar to the ones implicated in the acute hepatotoxicity (Milutinović et al., 2003). More recently, it was shown that even in the absence of renal injury, the acute exposure of rats (Moreno et al., 2005) and the sub-chronic exposure of mice (Andrinolo et al., 2007) to hepatotoxic doses of MCLR, induces a decrease of antioxidant defences and an increase of lipoperoxidation in kidneys. These results suggest that the nephrotoxicity of microcystins might be masked under severe acute hepatotoxic exposure but can be manifested after the hepatic effects have been overwhelmed or under a sub-acute or chronic exposure to lower toxin doses.

Additionally, Gaudin et al. (2008) reported that MCLR induces genotoxic effects (DNA damage by the Comet Assay) in mice kidney *in vivo*. Our preliminary data on the genotoxic potential of MCLR on Vero cells also showed that LMECYA 7 extract induces micronucleous formation, suggesting an aneugenic or clastogenic activity of microcystins (Dias et al., 2008).

Data presented here, together with those reported by others, point out the need to further clarify the effects of chronic exposure to low doses of MCLR at the kidney level, namely to what concerns the potential genotoxic and carcinogenic effects.

Acknowledgements

To Portuguese Foundation for Science and Technology for grants SFRH/BD/10585/2002 attributed to Elsa Dias. To National Health Institute, Portugal, for a master fellowship attributed to Mariana Andrade. To Institute for Medicines and Pharmaceutical Sciences (I-Med) and to Centre for Research on Human Molecular Genetics (CIGMH).

Conflict of interest

The authors declare that there are no conflicts of interest.

References

Andrinolo, D., Sedan, D., Telese, L., Aura, C., Masera, S., Giannuzzi, L., Marra, C.A., Alaniz, M.J.T., 2007. Hepatic recovery after damage produced by sub-chronic intoxication with the cyanotoxin microcystin-LR. Toxicon 51 (3), 457–467.

Aune, T., Berg, K., 1986. Use of freshly prepared rat hepatocytes to study toxicity of blooms of the blue-green algae *Microcystis aeruginosa* and Oscillatoria Agardhii. J. Toxicol. Environ. Health 19, 325–336.

Boaru, D.A., Dragos, N., SalasSchirmer, K., 2006. Microcystin-LR induced cellular effects in mammalian and fish primary hepatocytes cultures and cell lines: A comparative study. Toxicology 218, 134–148.

Borenfreund, E., Puerner, J., 1985. Toxicity determined in vitro by morphological alterations and neutral red absorption. Toxicol. Lett. 24, 119–124.

Botha, N., Gehringer, M.M., Downing, T.G., Venter, M., Shepard, E.G., 2004. The role of microcystin-LR induction of apoptosis and oxidative stress in CaCo2 cells. Toxicon 43, 185–192.
Boyer, J.L., Hagenbuch, B., Ananthanarayanan, M., Suchy, F., Stieger, B., Meier, P.J., 1993. Phylogenic and ontogenic expression of hepatocellular bile acid transport. Proc. Natl. Acad. Sci 90, 435–438.
Chong, M., Gu, K., Lam, P., Yang, M., Fong, W., 2000. Study on the cytotoxicityof microcystin-LR on cultured cells. Chemosphere 41, 143–147.
Chorus, I., Falconer, I.R., Salas, H.J., Bartram, J., 2000. Health risks caused by freshwater cyanobacteria in recreational waters. J. Toxicol. Environ. Health, Part B 3, 323–347.
Dias, E., Pereira, P., Batoréu, M.C.C., Jordan, P., Silva, M.J., 2008. Cytotoxic and genotoxic effects of microcystins in mammalian cell lines. In: Moestrup, Ø. (Ed.), Proceedings of the 12th International Conference on Harmful Algae. International Society for the Study of Harmful Algae and Intergovernmental Oceanographic Commission of UNESCO, Copenhagen, pp. 282–285.
Duy, T.N., Lam, P.K.S., Shaw, G.R., Connell, D.W., 2000. Toxicology and risk assessment of freshwater cyanobacterial (blue-green algal) toxins in water. Rev. Environ. Contam. Toxicol. 163, 113–186.
Eriksson, J.E., Paatero, G.I.L., Meriluoto, J.A.O., Codd, G.A., Kass, G.E.N., Nicotera, P., Orrenius, S., 1989. Rapid microfilament reorganization induced in isolated rat hepatocytes by microcystin-LR, a cyclic peptide toxin. Exp. Cell Res. 185, 86–100.
Falconer, I.R., Yeung, D.S., 1992. Cytoskeletal changes in hepatocytes induced by microcystis toxins and their relation to hyperphosphorilation of cell proteins. Chem. Biol. Interact. 81, 181–196.
Falconer, I.R., 2007. Cyanobacterial toxins present in *Microcystis aeruginosa* extracts – More than microcystins!. Toxicon 50, 585–588.
Fischer, W.L., Altheimer, S., Cattori, V., Meier, P.J., Dietrich, D.R., Hagenbuch, B., 2005. Organic anion transporting polypeptides expressed in liver and brain mediate uptake of microcystin. Toxicol. Appl. Pharmacol. 203, 257–263.
Funari, E., Testai, E., 2008. Human health risk assessment related to cyanotoxins exposure. Crit. Rev. Toxicol. 38, 97–125.
Gaudin, J., Huet, S., Jarry, G., Fessard, V., 2008. *In vivo* DNA damage induced by the cyanotoxin microcystin-LR: comparison of intraperitonial and oral administration by use of the comet assay. Mutat. Res. 652, 65–71.
Grabow, W., Randt, W., Prozesky, O., Scott, W., 1982. *Microcystis aeruginosa* toxin: cell culture toxicity, hemolysis and mutagenicity assays. Appl. Environ. Microbiol. 43 (6), 1425–1433.
Gupta, N., Pant, S.C., Vijayaraghavan, R., Rao, L., 2003. Comparative toxicity evaluation of cyanobacterial cyclic peptide toxin microcystin variants (LR, RR, YR) in mice. Toxicology 188, 285–296.
Hagenbuch, B., Meier, P.J., 2003. The superfamily of organic anion transporting polypeptides. Biochim. Biophys. Acta 1609, 1–18.
Harada, K., 1996. Chemistry and detection of microcystins. In: Watanabe, M., Harada, K., Carmichael, W.W., Fujiki, H. (Eds.), Toxic Microcystis. CCR Press, NY, pp. 103–148.
Khan, S.A., Ghosh, S., Wickstrom, M., Miller, L.A., Hess, R., Haschek, W.M., Beasley, V.R., 1995. Comparative pathology of microcystin-LR in cultured hepatocytes, fibroblasts and renal epithelial cells. Nat. Toxins 3, 119–128.
Legrand, C., Bour, J.M., Jacob, C., Capiaumont, J., Martial, A., Marc, A., Wudtke, M., Kretzmer, G., Demangel, C., Duval, D., 1992. Lactate dehydrogenase (LDH) activity of the cultured eukariotic cells as a marker of the number of dead cells in the medium. J. Biotechnol. 25, 231–243.
McDermott, C.M., Nho, C.W., Howard, W., Holton, B., 1998. The cyanobacterial toxin, microcystin-LR, can induce apoptosis in a variety of cell types. Toxicon 36 (12), 1981–1986.
Mereish, K.A., Solow, R., 1990. Effect of antihepatotoxic agents against microcystin-LR toxicity in cultured rat hepatocytes. Pharm. Res. 7 (3), 256–259.
Moreno, I., Pichardo, S., Jos, A., Gómez-Amores, L., Mate, A., Vasquez, C.M., caméan, A.M., 2005. Antioxidante enzyme activity and lipid peroxidation in liver and kidney of rats exposed to microcystin-LR, administred intraperitoneally. Toxicon 45, 395–402.
Mossmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 53–63.
Milutinović, A., Sedmark, B., Horvat-ŽnidarŠić, I., Šuput, D., 2002. Renal injuries induced by chronic intoxication with microcystins. Cell Mol. Biol. Lett. 7 (1), 139–141.
Milutinović, A., Živin, M., Zorc-Plesković, R., Sedmark, B., Šuput, D., 2003. Nephrotoxic effects of chronic administration of microcystins-LR and -YR. Toxicon 42, 281–288.
Nobre, A.C.L., Jorge, M.C.M., Menezes, D.B., Fonteles, M.C., Monteiro, H.S.A., 1999. Effects of microcystin-LR in isolated perfused rat kidney. Braz. J. Med. Biol. Res. 32, 985–988.
Pereira, P., Onodera, H., Andrinolo, D., Franca, S., Araújo, F., Lagos, N., Oshima, Y., 2001. Co-occurrence of microcystins and saxitoxins Montargil reservoir, Portugal. In: Hallegraeff, G.M., Blackburn, S.I., Bolch, C.J., Lewis, R.J. (Eds.), Harmful Algal Blooms. IOC, UNESCO, pp. 108–111.
Philips, H.J., 1973. Dye exclusion tests for cell viability. In: Kruse, P.F., Patterson, M.K. (Eds.), Tissue Culture: Methods and Applications. Academic Press, NY, pp. 406–408.
Pichardo, S., Jos, A., Zurita, J.L., Salguero, M., Camean, A.M., Reppeto, G., 2005. The use of the fish cell lines RTG-2 and PLHC-1 to compare the toxic effects produced by microcystins LR and RR. Toxicology in Vitro 19, 865–873.
Pietsch, C., Wiegrand, C., Ame, M.V., Nicklisch, A., Wunderlin, D., Pflugmacher, S., 2001. The effects of a cyanobacterial crude extract on different aquatic organisms: evidence for cyanobacterial toxin modulating factors. Environ. Toxicol. 16, 535–542.
Runnegar, M.T.C., Gerdes, R.G., Falconer, I.R., 1991. The uptake of the cyanobacterial hepatotoxin microcystin by isolated rat hepatocytes. Toxicon 29 (1), 43–51.
Runnegar, M.T.C., Falconer, I.R., 1982. The *in vivo* and *in vitro* biological effects of the peptide hepatotoxin from the blue-green alga *Microcystis aeruginosa*. S. Afr J. Sci. 78, 363–366.
Sivonen, K., Jones, G., 1999. Cyanobacterial toxins. In: Chorus, I., Bartram, J. (Eds.), Toxic cyanobacteria in water: a guide to their public health consequences, monitoring and management. E & FN Spon, London, pp. 41–111.
Skulberg, R., Skulberg, O.M., 1990. Forskning med algekulturer NIVAs kultursampling av alger. NIVA, , Norway. pp.32.
Thompson, W.L., Allen, M.B., Bostian, K.A., 1987. The effects of microcystin on monolayers of primary rat hepatocytes. In: Gopalakrisnakonef, P., Tan, C.K. (Eds.), Progress in venom and toxin research. National University of Singapore, Singapore, pp. 725–731.
Ueno, Y., Nagata, S., Tsutsumi, T., Hasegawa, A., Watanabe, M.F., Park, H.D., Chen, G.C., Chen, G., Yu, S.Z., 1996. Detection of microcystins, a blue-green algal hepatotoxins, in drinking water sampled in Haimen and Fusui, endemic areas of primary liver cancer in China, by highly sensitive immunoassay. Carcinogenesis 17 (6), 1317–1321.
WHO, 1998. Guidelines for Drinking Water Quality, Second ed. World Health Organization, Geneva. Addendum to Volume 2, Health Criteria and other supporting information.
Yoshizawa, S., Matsushima, R., Watanabe, M.F., Harada, H., Ichihara, A., Carmichael, W.W., Fujiki, H., 1990. Inhibition of protein phosphatases by microcystis and nodularin associated with hepatotoxicity. J. Cancer Res. Clin. Oncol. 116, 609–614.

CAPÍTULO 4

Alverca, E., et al., Morphological and ultrastructural effects of microcystin-LR from *Microcystis aeruginosa* extract on a kidney cell line, Toxicon (2009), doi: 10.1016/j.toxicon.2009.04.014.

Toxicon xxx (2009) 1–12

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Morphological and ultrastructural effects of microcystin-LR from *Microcystis aeruginosa* extract on a kidney cell line

E. Alverca [a,*], M. Andrade [a], E. Dias [a,b,c], F. Sam Bento [a], M.C.C. Batoréu [c], P. Jordan [b], M.J. Silva [b], P. Pereira [a]

[a] National Institute of Health Dr. Ricardo Jorge, Department of Environmental Health, Av. Padre Cruz, 1649-016 Lisbon, Portugal
[b] National Institute of Health Dr. Ricardo Jorge, Department of Genetics, Av. Padre Cruz, 1649-016 Lisbon, Portugal
[c] University of Lisbon, Faculty of Pharmacy, I-Med, Laboratory of Toxicology, Av. Prof. Gama Pinto, 1649-019 Lisbon, Portugal

ARTICLE INFO

Article history:
Received 16 February 2009
Received in revised form 13 April 2009
Accepted 14 April 2009
Available online xxxx

Keywords:
Microcystin-LR
Vero cells
Cytotoxicity
Fluorescence microscopy
TEM

ABSTRACT

The aim of this study was to examine the toxic effects of a microcystin-LR (MCLR)-containing cyanobacteria extract on the subcellular organization of a kidney cell line (Vero-E6). Cells were exposed to different MCLR concentrations (1.3–150 μM) for 24, 48 and 72 h and two cytotoxicity assays were performed. This information was combined with the analysis of lysosomal, mitochondrial and cytoskeleton integrity and with an ultrastructural study. Biochemical and microscopic data revealed a good agreement and demonstrated that cellular response to MCLR is dependent on the dose/exposure time. Cell viability decayed markedly after 24 h of exposure to toxin concentrations greater than 30 μM. Furthermore, it was demonstrated that lysosome destabilization precedes mitochondria dysfunction. The ultrastructural analysis showed that mild toxin incubation conditions induce endoplasmic reticulum (ER) vacuolization and assembly of large autophagic vacuoles, suggesting that autophagy is an early cellular response to the toxin. After exposure to higher MCLR doses, the number of apoptotic cells increased, as identified by microscopic observations and confirmed with TUNEL assay. Additionally, drastic exposure conditions induced the increase of necrotic cells. These results suggest that the ER is the primary microcystin target in Vero cells and that autophagy, apoptosis and necrosis are induced in a dose- and time-dependent manner.

1. Introduction

Increasing eutrophication of freshwater plays an important role in the development of cyanobacterial blooms. Cyanobacteria are distributed worldwide and have the ability to produce several types of toxins, the most frequent of which are microcystins, a family of potent hepatotoxins (Carmichael, 1994). Among these toxins, the most common and toxic variant is microcystin-LR (MCLR), which has been responsible for acute poisoning of humans and animals (Chorus et al., 2000; Duy et al., 2000). An association between the increase of hepatocarcinoma incidence in populations and exposure to water frequently contaminated with toxic strains of *Microcystis aeruginosa* was also reported (Ueno et al., 1996). In fact, MCLR is considered a tumor promoter (Nishiwaki-Matsushima et al., 1992; Humpage et al., 2000) and is classified by the International Agency for Research on Cancer as possibly carcinogenic to humans (IARC, 2006).

The acute hepatotoxicity of microcystins has been attributed to their activity as potent inhibitors of protein phosphatases PP1 and PP2A (Honkanen et al., 1990; Matsushima et al., 1990). This inhibition induces the

* Corresponding author. Tel.: +351 217519393; fax: +351 217526400.
E-mail address: Elsa.Alverca@insa.min-saude.pt (E. Alverca).

0041-0101/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.toxicon.2009.04.014

hyperphosphorylation of cytoskeleton proteins, disassembly of its components, resulting in hepatocyte deformation, dissociation and necrosis and ultimately, in intrahepatic hemorrhage and death (Bischoff, 2001; Carmichael, 1994). The effects of microcystins on cytoskeleton morphology and ultrastructure have been extensively documented for rodent liver cells (Billam et al., 2008; Toivola and Eriksson, 1999; Ito et al., 1997; Khan et al., 1995, 1996; Eriksson et al., 1990). Similar effects were also reported for fish (Pichardo et al., 2005; Li et al., 2001) and human (Batista et al., 2003) hepatocytes, rat kidney epithelial cells and skin fibroblasts (Khan et al., 1995, 1996) and rabbit embryo cells (Frangež et al., 2003).

More recently other subcellular effects have been reported, particularly those consistent with apoptosis. These include cell shrinkage, membrane blebbing and DNA fragmentation, which have been described for rodent or human hepatocytes (Bouaïcha and Maatouk, 2004; Ding et al., 2001; Mankiewicz et al., 2001; McDermott et al., 1998), human lymphocytes (Mankiewicz et al., 2001; Lankoff et al., 2004) and for some mammalian cell lines (Lankoff et al., 2003; McDermott et al., 1998; Rao et al., 1998). Additionally, the ability of microcystins to induce liver apoptosis *in vivo* has been already confirmed in studies with mouse (Weng et al., 2007; Chen et al., 2005; Hooser, 2000; Guzman and Solter, 1999) and fish (Li et al., 2005). Several cellular and molecular mechanisms have been proposed for MCLR-induced apoptosis, and the most widely accepted are those related with the mitochondria pathway and oxidative stress. It has been shown that MCLR causes the reactive oxygen species (ROS) overproduction and glutathione (GSH) depletion in mouse hepatocytes (Ding and Ong, 2003). Furthermore, it has been demonstrated that MCLR induces changes in mitochondrial membrane potential (MMP) and permeability with the consequent release of mitochondrial cytochrome *c* and Ca^{2+} (Ding et al., 2000a, 2001), activation of calpin and Ca^{2+}/calmodulin-dependend kinase II (Fladmark et al., 2002), impairment of the electron transport chain as well as mitochondria ultrastructure changes (Zhao et al., 2008). Additionally, it has been proposed that other mechanisms might also be involved in the microcystin-induced apoptosis such as the activation of caspases (Fladmark et al., 1999), altered expression of pro-apoptotic proteins from Bcl-2 family and of *p53* gene (Billam et al., 2008; Fu et al., 2005; Weng et al., 2007). Besides the changes in mitochondria functions and structure, few authors have also reported the involvement of lysosomes (Boaru et al., 2006; Li et al., 2007) in the MCLR-mediated apoptosis.

Despite the increasing recognition of the toxic effects of microcystins, the underlying mechanisms of toxicity are still poorly characterized, particularly in non-liver cell models. The organ specificity of microcystins is due to the selective uptake of the toxin by the Organic Anion Polypeptide Transporter (OAPT), a family of transport proteins that are selectively expressed by hepatocytes (Fischer et al., 2005). Microcystins are excreted mainly by the liver, but a small proportion is also eliminated through the urine (9%) (Bischoff, 2001). Some papers have described toxic effects of microcystins in kidney (Nobre et al., 1999; Milutinović et al., 2003; Gaudin et al., 2008) and intestine cells (Botha et al., 2004; Gaudin et al., 2008), showing that, besides the liver, other organs might also be targets for microcystins. In previous studies (Dias et al., 2008, 2009), we showed that MCLR is able to induce cytotoxicity in the monkey kidney cell line Vero-E6. However, its impact on subcellular organization has never been characterized in this cell line. In the present work, we evaluated the effects of a semi-purified MCLR-containing cyanobacterial extract in Vero cells at morphologic and ultrastructural levels using both Fluorescence and Transmission Electron Microscopy (TEM) approaches, respectively.

2. Material and methods

2.1. Cyanobacteria extracts

The MCLR-containing extract was obtained from a toxic *M. aeruginosa* strain (LMECYA 113), isolated from a natural bloom (Montargil reservoir, Portugal) and maintained in monoalgal, free of eukaryotes, non-axenic laboratory cultures. The characterization of microcystins production in this strain was previously done by Valério et al. (in press). HPLC-DAD revealed that LMECYA 113 chromatogram exhibited only one peak with the typical absorbance spectrum of microcystins and the retention time of the MCLR variant. LMECYA 113 grew in 2 L plankton light reactors (Aqua-Medic, Bissendorf, Germany) containing Z8 medium (Skulberg and Skulberg, 1990) under continuous aeration, with a 14/10 h L/D cycle (light intensity 16 ± 4 μE m^{-2} s^{-1}) at 20 ± 1 °C. Cells harvested during late exponential growth phase were lyophilized in a freeze drier (Micromodul Y10, Savant, NY, USA) and extracted with 75% methanol (10 mL/100 mg dw) 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 submitted to rotary evaporation at 35 °C (Buchi-R, Flawil, Switzerland) to eliminate the alcoholic fraction. The resulting aqueous extract was cleaned-up by solid phase extraction on Sep-Pak C18 cartridges (500 mg Waters, Massachusetts, USA) previously conditioned with 20 mL of methanol and equilibrated with 20 mL of distilled water. The microcystin-LR containing fraction was eluted with methanol at 80% (v/v) and evaporated to dryness. The solid residue was re-suspended in 25 mM acetic acid and manually injected into a Bio-Gel P2 (40–90 μm, Bio-Rad Inc., CA, USA) packed preparative column (Amersham Biosciences, XK 26/40, i.d./length). The mobile phase consisted of 25 mM acetic acid, and the flow rate was set at 1 mL min^{-1} (Knauer Wellchrom K-120 pumps, Germany). Elution fractions (5 mL) were collected on a fraction collector (Bio-Rad Mod. 2110, CA, USA) and analyzed by HPLC-DAD according to the ISO standard method 20179 (ISO 2005). The purity of the MCLR fractions (>85%) was assessed by quantifying the closely retaining impurities (peaks eluting within 4 min before and after toxin peak) by fitting them to the MCLR calibration curve (Ramanan et al., 2000). The later was constructed by analyzing commercially available MCLR standards (Alexis Biochemicals, CA, USA). MCLR-containing fractions were evaporated to dryness and re-suspended in MEM culture

Please cite this article in press as: Alverca, E., et al., Morphological and ultrastructural effects of microcystin-LR from *Microcystis aeruginosa* extract on a kidney cell line, Toxicon (2009), doi:10.1016/j.toxicon.2009.04.014

medium (see following section). The final extracts were sterilized by filtration on 0.22 μm syringe filters and kept at −20 °C until use.

2.2. Vero cell line culture

African green monkey *Cercopithecus aethiops* kidney cells (Vero-E6, FTV6, Vircell, Granada, Spain) were maintained in 25 cm^2 flasks (Nunc, Roskilde, Denmark) in Minimum Essential Medium (MEM), supplemented with 1% essential amino acid, 1% sodium pyruvate and 10% Foetal Bovine Serum (FBS), at 37 °C in a 5% CO_2 humidified incubator. All culture media and supplements were purchased from Gibco BRL (Paisley, UK). Exponential growing cells were trypsinized and counted using a haemocytometer by the trypan blue exclusion method (Philips, 1973). Densities of: (1) 1×10^4 cells were seeded in 96-well microtitre plates (Sarstedt, Newton, USA) for the cell viability assays; (2) 3.5×10^4 cells were seeded on coverslips in sterile 4-chamber slides (Nunc) for morphological evaluation by fluorescence microscopy and apoptosis analysis by the TUNEL assay; (3) 1×10^5 cells were seeded in 6-well tissue culture plates (Nunc) for ultrastructure analysis by Transmission Electron Microscopy (TEM).

2.3. Toxin exposure

For the viability assays, the culture medium was replaced by several dilutions of the LMECYA 113 extract containing 1.3–150 μM of MCLR and cells were exposed for 24, 48 and 72 h. A similar procedure was conducted for the microscopic analysis. After medium discharge the cells were exposed for 24 h and 48 h to the LMECYA 113 extract containing 5, 20 and 40 μM of MCLR for the fluorescent morphologic assays, 20 and 40 μM of MCLR for the TUNEL assay and 5, 10, 20, 30, 40 and 75 μM of MCLR for the TEM analysis. The negative controls for all the exposure conditions consisted of cells grown in MEM culture medium.

2.4. Cytotoxicity assays

The effects on cell viability were evaluated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and NR (neutral red) methods described by Mosmann (1983) and Borenfreund and Puerner (1985), respectively. Briefly, after toxin exposure, the cells were washed with PBS and incubated for 3 h with the MTT (0.5 mg mL^{-1}) or NR (50 μg mL^{-1}) solutions prepared in MEM culture medium. For MTT assay, a 100 μl of acidified propan-2-ol solution (0.04 M HCl) was added to dissolve MTT–formazan crystals and after shaking for 15 min, absorbance was recorded at 570 nm using a Multiscan Ascent spectrophotometer (Labsystems, Helsinki, Finland). For the NR assay, the cells were washed twice with PBS and the intracellular neutral red was extracted with 50% aqueous ethanol containing 1% acetic acid. The plates were shaked for 20 min in the dark and the absorbance was measured at 540 nm. MTT was purchased from Calbiochem (Darmstadt, Germany) and NR from Merck (Darmstadt, Germany).

2.5. Fluorescence microscopy

2.5.1. Lysosomes and mitochondria

Acridine Orange (AO) and Rhodamine (Rh)-123 (Molecular Probes, Oregon, USA) were used as fluorescent probes for lysosome and mitochondria staining, respectively. Cells were incubated for 10 min with 10 μM AO or 15 μg mL^{-1} Rh-123, thoroughly washed with PBS or culture medium, and observed under fluorescent filters ($\lambda = 487$ nm for AO and $\lambda = 505$ nm for Rh-123).

2.5.2. Actin filaments

Cells were fixed with freshly prepared 3.7% paraformaldehyde (PFA) in CBS buffer (10 mM MES, 138 mM KCl, 3 mM $MgCl_2$, 2 mM EGTA, 0,32 M sucrose, pH 6.9) for 15 min. The cells were permeabilized with 0.5% Triton X-100 in phosphate-buffered saline (PBS) for 3 min, and then blocked with 2% bovine serum albumin in PBS for 10 min at room temperature. The microfilaments (MFs) and nuclei were then stained with Alexa Fluor® 488 Phalloidin (30 U/mL) (Molecular Probes, Oregon, USA) and 1 μg mL^{-1} DAPI (4′,6-diamidino-2-phenylindole), respectively.

2.5.3. Microtubules

The cells were first washed with a microtubule stabilizing buffer (MTB) containing 50 mM PIPES, 2 mM EGTA, 2 mM $MgSO_4 \cdot 7H_2O$ (pH 6.1). Afterwards they were fixed with freshly prepared 3.7% PFA in MTB for 45 min, washed twice with the same buffer and permeabilized with 0.5% Triton X-100 in PBS. The cells were then incubated with the primary antibody DM1A (dilution 1:50) (Sigma, MO, USA) for 90 min. Antigen–primary antibody complex visualization was achieved with an anti-mouse secondary antibody conjugated to Alexa Fluor® 488 (dilution 1:200). The preparations were then counterstained with 1 μg mL^{-1} DAPI for nuclei visualization.

For both microtubule (MT) and MFs staining, the coverslips were mounted on glass slides with a drop of Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). All preparations were observed under an Olympus BX60 microscope and microphotographs captured with a CCD camera (Olympus DP11).

2.6. Transmission Electron Microscopy

After the exposure treatments, both supernatant and trypsinized adherent cells were centrifuged at 209 × *g* for 5 min. The supernatant was removed and the pellet was fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer plus 2.5 mM $CaCl_2$ (pH 7.2), for 90 min at room temperature. Cells were post-fixed in an aqueous solution of 1% OsO_4 and 1.5% $K_3Fe(CN)_6$, for 90 min; contrasted with 1% uranyl acetate for 1 h, dehydrated in ethanol and embedded in Spurr's resin (EMS, Washington, USA). Ultrathin sections were contrasted with saturated uranyl acetate and lead citrate and examined under a Morgagni 268D EM. Digital images were acquired with a CCD MegaView (SIS, Münster, Germany).

Please cite this article in press as: Alverca, E., et al., Morphological and ultrastructural effects of microcystin-LR from *Microcystis aeruginosa* extract on a kidney cell line, Toxicon (2009), doi:10.1016/j.toxicon.2009.04.014

2.7. TUNEL assay

The apoptotic effect was determined using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) technique. Labeling of DNA strand breaks by TUNEL was performed using a commercial kit (*In situ* Cell Death Detection Kit, Roche Diagnostics, Indianapolis, USA), according to the manufacturer's instructions. This assay was applied on both adherent cells on the coverslips and supernatant cells, which after MCLR treatment detached from growth surface to the exposure medium. After labeling, cells were counterstained with 1 $\mu g\,mL^{-1}$ DAPI and mounted on slides with Vectashield. Negative (no TdT) and positive (DNase I treatment) controls were prepared as indicated in the TUNEL kit.

2.8. Statistical analysis

In cell viability assays, each treatment condition was assayed in triplicate. Results are expressed as mean ± standard deviation of the percentage of that found in control cultures. The statistical differences were analyzed with a factorial ANOVA and Tukey's test (Statistica software 7.0). Cell viability data were fitted by multiple regression to polynomial curves ($p < 0.0001$) and the EC_{50} value for each exposure time was calculated by interpolation of those polynomial dose–response curves. For TUNEL assay, experiments were performed in duplicate. For each exposure condition, apoptotic nuclei were counted in a total of 500 cells. The data were expressed as percentage of apoptotic nuclei (apoptotic index) and presented as mean ± standard deviation. The statistical differences were analyzed with Qui-square, Qui-square for Trend and Fisher method using the Statistica software 7.0. The level of statistical significance employed in all cases was $p < 0.05$.

3. Results

3.1. Cell viability

The cytotoxic effect of MCLR on Vero cell line was assessed by the reduction of MTT and the uptake of NR by viable cells (Fig. 1). A significant dose- and time-dependent loss of cell viability ($p < 0.0001$, factorial ANOVA) was observed in both assays. For the 24 h treatment, the cell viability started to decrease significantly at 30 μM of MCLR. After 48 h of exposure, the cell viability became significantly ($p < 0.05$, Tukey's test) distinct from control at lower MCLR concentrations (20 μM by the MTT assay, 5 μM by the NR assay). At 72 h, a similar trend was observed and by the NR assay only 10% of cells were viable above 10 μM MCLR. Regardless the duration of exposure, the EC_{50} values calculated from the MTT results were always higher (1.3–2.7 times) than those obtained by the NR assay (Fig. 2). These differences might be attributed to distinct sensitivities of NR and MTT assays, or might reflect the end-points assessed by those methods. Considering that the MTT and NR assays evaluate mitochondrial and lysosomal integrity, respectively, MCLR seems to affect lysosomes earlier than mitochondria in Vero cells.

3.2. Cell morphology

To further investigate the mechanisms underlying the MCLR-mediated cytotoxicity, fluorescence techniques were used to study lysosomal, mitochondrial and cytoskeleton integrity.

In untreated Vero cells, the Acridine Orange (AO) staining revealed the presence of a large number of red fluorescent lysosomes, uniformly distributed within the cytoplasm (Fig. 3A). Cells exposed to 5 μM MCLR for 24 h presented enlarged lysosomes (Fig. 3B), effect seen before viability decrease. Following 20 and 40 μM MCLR (24 h) exposure, the presence of enlarged lysosomes was also observed. However, it was evident a drastic reduction on the number of intact lysosomes and an increase of green cytosolic fluorescence, reflecting the leakage of lysosomal AO to the cytoplasm (Fig. 3C, D). Doubling the duration of the exposure to the toxin (48 h), there was a reduction in the number of intact lysosomes, their redistribution in the cell and an increase in cytoplasmic green fluorescence. These effects were detected in cells exposed to 5 μM MCLR (Fig. 3E), but became more evident with higher MCLR concentrations (data not shown).

Mitochondrial integrity was evaluated by Rh-123 incorporation into viable mitochondria. In control cells, labeled mitochondria appeared as a reticular network with a perinuclear distribution (Fig. 3F). The same pattern was observed in cells treated with 5 μM MCLR for 24 h (Fig. 3G). Higher MCLR doses induced mitochondria structural damage in most of the cells as shown by the diffusion of fluorescence to the cytosol (Fig. 3H, I). As shown in Fig. 3J, this process of mitochondrial degradation was also noted in some cells exposed to 5 μM MCLR for 48 h, although it was more pronounced in cells treated with higher MCLR concentrations (20 μM and 40 μM, data not shown).

To analyze the MCLR effects on the cytoskeleton, immunolabelling of microfilaments and microtubules was used (Fig. 4).

Untreated cells displayed a highly organized mesh of cortical actin filaments, prominent stress fibers and dense actin bundles at the cell periphery (Fig. 4A). No changes to this structure were observed in cells exposed to 5 μM MCLR, for 24 h (data not shown), while treatments with higher MCLR concentrations caused the progressive disassembly of actin filaments, as well as the shortening or disappearance of stress fibers (Fig. 4B, C). Similar dose-dependent effects were observed in cells exposed to the toxin for 48 h. As can be seen in Fig. 4D, cells treated with 5 μM MCLR for 48 h presented already short stress fibers that seem to be collapsed into several cytoplasmic points.

Microtubule immunolabelling of control cells, revealed the presence of a complex network of filaments radiating from nucleus to the cell periphery (Fig. 4E). Again, at 24 h treatments, toxin doses higher than 5 μM (20 μM and 40 μM) induced the microtubule aggregation, forming intense green bundles (Fig. 4F, G). Some cells exposed to 40 μM MCLR, also showed partially collapsed microtubules in the perinuclear region (Fig. 4G). Similar effects were observed when cells were subjected to 48 h treatments, but at lower toxin concentrations (Fig. 4H). Longer exposure time also induced slightly microtubule disassembly, as shown by the presence of fluorescent dots (Fig. 4H).

Please cite this article in press as: Alverca, E., et al., Morphological and ultrastructural effects of microcystin-LR from *Microcystis aeruginosa* extract on a kidney cell line, Toxicon (2009), doi:10.1016/j.toxicon.2009.04.014

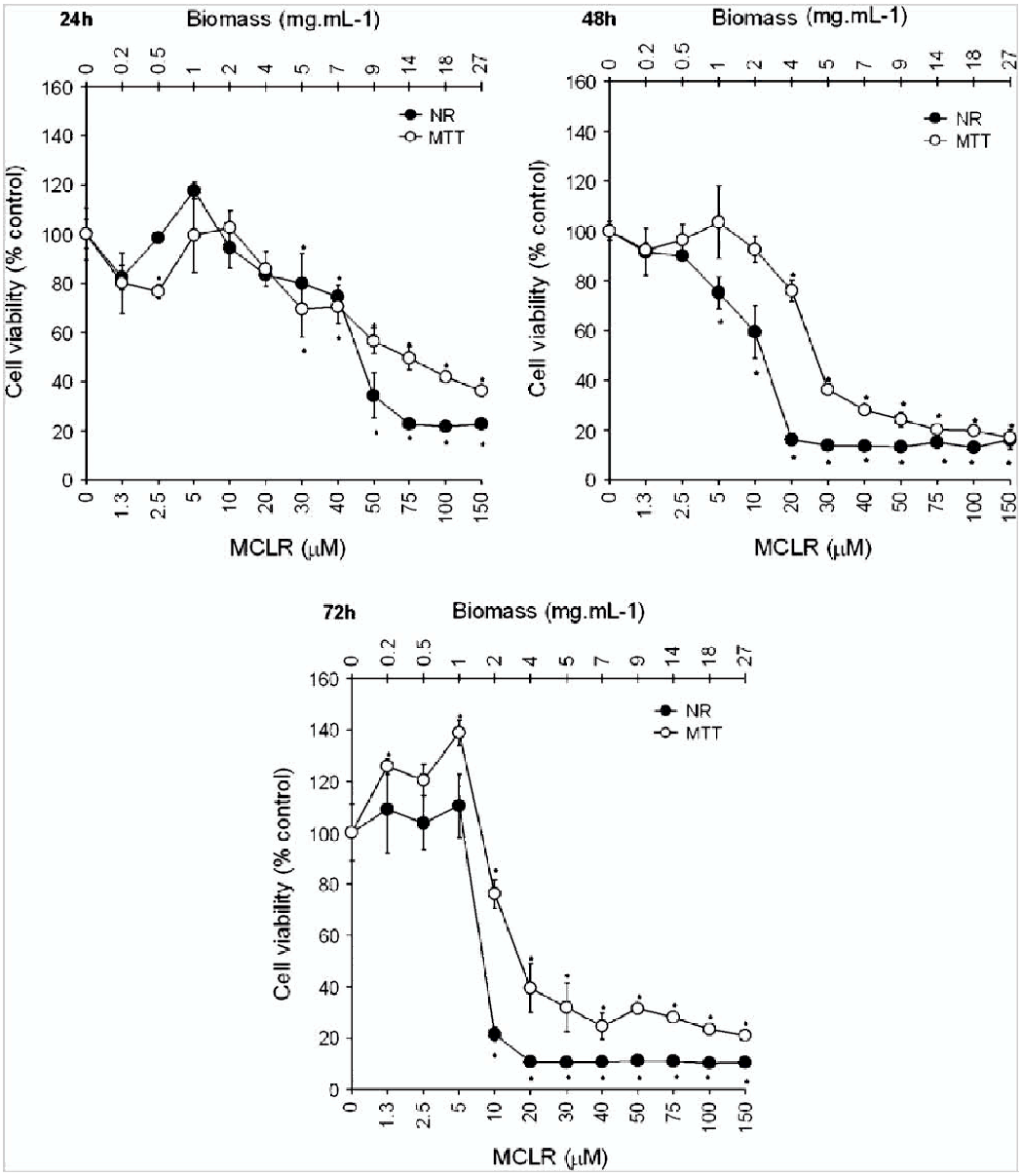


Fig. 1. Viability (MTT and NR assays) of Vero cells exposed for 24, 48 and 72 h to LMECYA 113 extract containing 1.3–150 μM of MCLR. The secondary *x*-axis represents the corresponding biomass concentration (mg dw mL^{-1}). Results are expressed as mean % ± SD of three replicates relative to control. * Indicates a significant difference from control, $p < 0.05$.

3.3. Cell ultrastructure

Transmission Electron Microscopy analysis was performed to determine ultrastructural changes occurring during MCLR treatments (Fig. 5). After 24 and 48 h of culture, control Vero cells showed the typical ultrastructural organization as depicted in Fig. 5A. After 24 h treatment with 5 μM MCLR, some cells presented one or more large digestive vacuoles with cytoplasmic contents and frequently showing signs of fusion with lysosomes (Fig. 5B). In some cases, it was also evident the swelling of the endoplasmic reticulum (ER) cisterns (Fig. 5C). Doubled exposure time additionally induced the Golgi apparatus vacuolization (Fig. 5D). Both cell blebbing and Golgi apparatus vacuolization were also observed in cells exposed to the toxin for 24 h, but with 10 μM and higher MCLR doses (Fig. 5E, F). Mitochondrial ultrastructure began to be altered in cells exposed to 10 μM MCLR for 48 h (Fig. 5G). They appeared reduced in size and with a condensed matrix. Similar effects could also be observed after 24 h of incubation but with higher MCLR concentrations (data not shown). The presence of cells with condensed chromatin and amorphous cytoplasm, ultrastructurally consistent with apoptotic cells, was more frequently detected with longer (48 h) treatments, using 20 μM or higher toxin concentrations (Fig. 5H, J). With this treatment condition there were also non-apoptotic cells, presenting the previously mentioned organelle injuries, as well an abnormal microtubules aggregation (Fig. 5I).

In cultures treated with 40 μM or higher MCLR concentrations, most of the cells showed drastic ultrastructural changes. Besides the condensed apoptotic cells, it

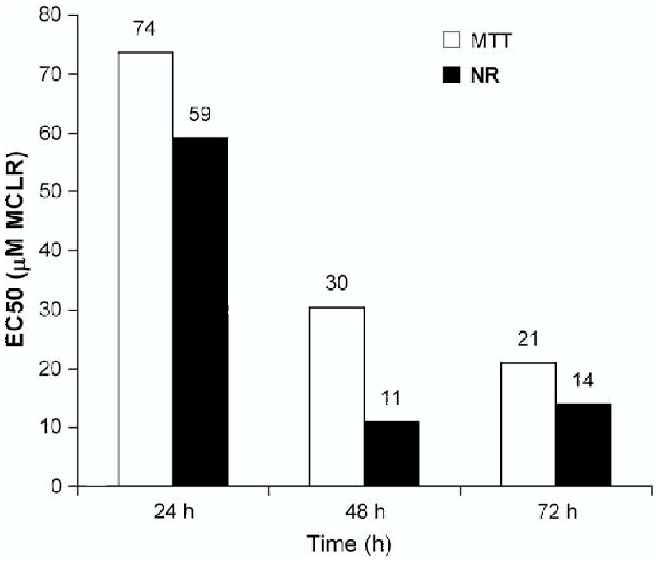


Fig. 2. EC_{50} values of MCLR after 24, 48 and 72 h of exposure of Vero cells to LMECYA 113 extract. EC_{50} represents the mean effective concentration of MCLR that induced the loss of 50% of cell viability determined by both MTT and NR assays, relative to negative control.

was evident the increase on the proportion of necrotic cells, especially after 48 h of incubation with the toxin. These cells were characterized by a generalized cytoplasmic disorganization and membrane disruption (Fig. 5L).

Cells exposure to the toxin, affected progressively different organelles in a dose- and a time-depending manner.

3.4. Apoptosis detection (TUNEL assay)

Quantification of MCLR-induced apoptotic cells by the TUNEL assay identified 4% of apoptotic cells after the 5 μM/24 h treatment (Fig. 6). Statistical analysis confirmed a significant dose- and time-dependent increase in apoptotic cells after treatments with MCLR ($p < 0.001$). Additionally, TUNEL assay results revealed that the occurrence of apoptotic cells in the supernatant was significantly superior when compared to the correspondent adherent cells.

4. Discussion

In previous studies we showed that the MCLR effects on Vero cells are highly dependent on dose/time of exposure, as well as on the cytotoxicity end-point. We also demonstrated that the cytotoxic effects of a MCLR-containing *M. aeruginosa* extract was similar to those induced by commercial MCLR, excluding potential toxicity from other eventual extract compounds (Dias et al., 2009). In this work, we used an extract from another MCLR-producer (*M. aeruginosa* strain LMECYA 113) prepared by the same purification procedure. This extract induced dose–response curves similar to those obtained with pure MCLR: 1 – cell viability decrease was highly dependent on the MCLR concentration/time of exposure; 2 – the cytotoxicity threshold of LMECYA 113 extract was 25 μM MCLR/24 h; 3 – the neutral red assay was the most sensitive to evaluate the MCLR-induced cytotoxicity.

The morphological and ultrastructural analysis of LMECYA 113-treated Vero cells, allowed us to clarify the cellular damages underlying the identified MCLR-induced cytotoxicity. According to the data from TEM studies, the ER is the most sensitive organelle to the MCLR toxic effects, probably representing the first intracellular target for this toxin. In fact, ER vacuolization was evident even at the mildest toxin treatment tested (5 μM/24 h). Similar effects were reported by Berg et al. (1988) and Li et al. (2001) using MCLR from crude cyanobacterial extracts in hepatocytes from rats and carp, respectively. These authors suggested that the mentioned structural effects were related with cytoskeleton modifications. However, our results from immunolabelling of microfilaments and microtubules showed no changes in these structures at this toxin concentration, suggesting a different cause. Cells exposed to higher incubation times or higher MCLR concentrations, displayed simultaneous vacuolization of the Golgi apparatus. This is a highly dynamic organelle, whose structure is partially dependent on the ER–Golgi transport equilibrium (Dinter and Berger, 1998; Lee and Linstedt, 1999). As described by Davidson et al. (1992) in a study done with ovary and kidney cell lines, this transport is inhibited as a consequence of MCLR action on PP2A activity, indicating a casual relationship between the ER and Golgi ultrastructural changes observed. Recent studies further suggested that the ER and the Golgi apparatus have detection and signaling stress mechanisms that are able to activate cell repair or death pathways (Maag et al., 2003; Hicks and Machamer, 2005), supporting the idea that these organelles may play an important role in cell response to MCLR.

Autophagy is a mechanism highly conserved from yeast to mammalian cells. This process is characterized by sequestration of cytosolic proteins and/or organelles in double membrane vesicles and their subsequent degradation by the cell's lysosomal system (Bröker et al., 2005). In our study, the presence of cells with prominent digestive vacuoles was frequently observed following MCLR treatment. Both TEM and AO incorporation observations revealed that these large autophagic vacuoles were predominantly present in cells grown under mild toxin exposure conditions. The formation of autophagic/digestive vacuoles in mouse hepatocytes has been previously reported by Dabholkar and Carmichael (1987), after a lethal injection of MCLR, suggesting that they were involved in the toxin neutralization. Additionally, studies developed in silver carp revealed an increment of secondary lysosomes after microcystin treatments, indicating that this might be a mechanism to eliminate or minimize the toxin impact on cells (Li et al., 2007). In fact, it has been proposed that in some mammalian cell lines the attempt to remove injured organelles through an autophagic process precedes the cells' commitment to death, namely by apoptosis (Bauvy et al., 2001). In our work, the observed stimulation of the autophagic cellular response under mild MCLR exposure conditions is compatible with a cell survival strategy. Furthermore, there is a growing body of evidence that the

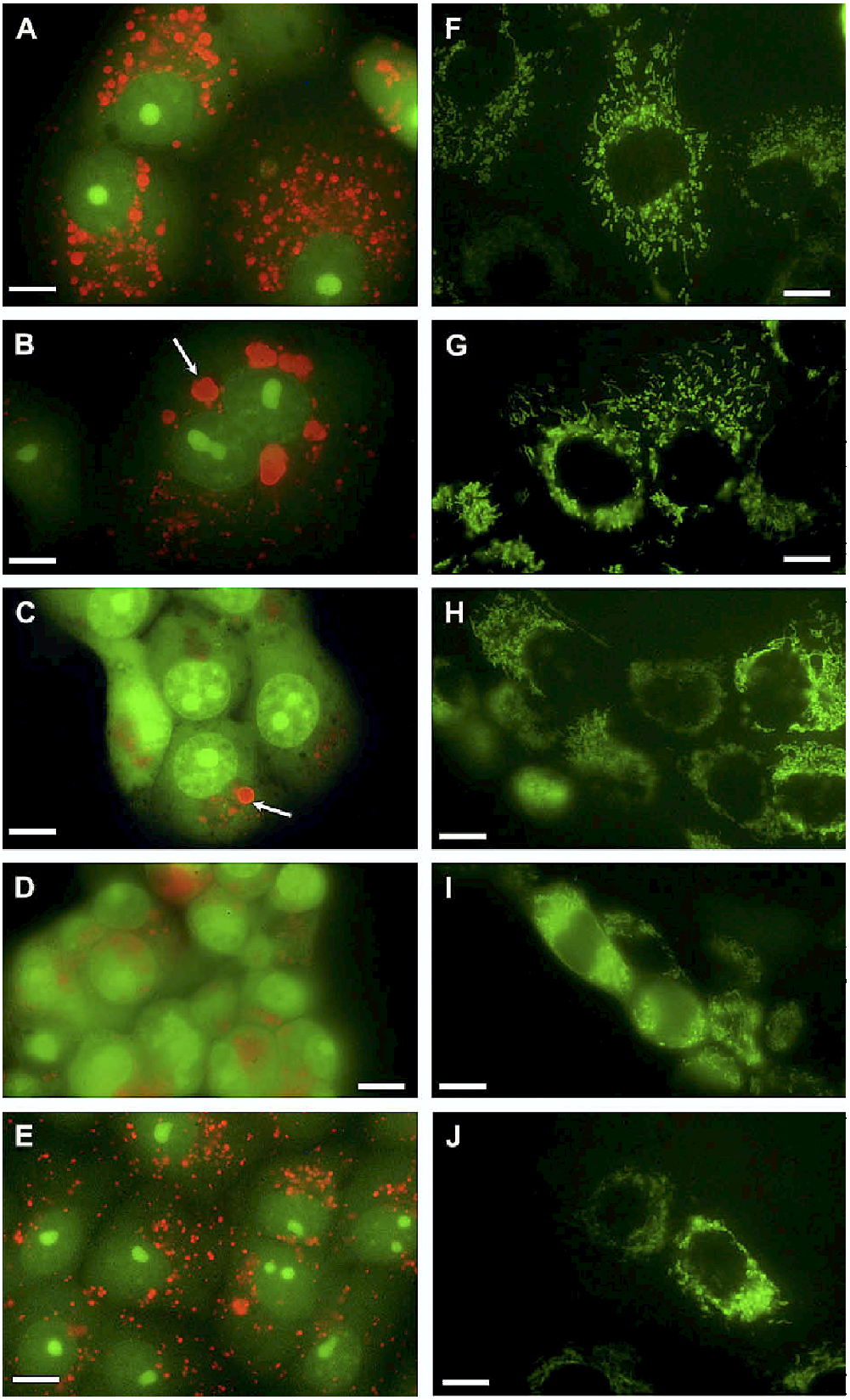


Fig. 3. Fluorescence microscopy analysis of Vero cells stained with AO (A–E) or Rh-123 (F–J) after treatments with MCLR. A. Control cells showing brightly labeled lysosomes dispersed within the cytoplasm. B. Note the presence of some swelled lysosomes (arrow) already after 24 h exposure to 5 μM MCLR. C. Cells treated with 20 μM for 24 h, with some enlarged lysosomes (arrow) and an increase of cytoplasmic and nuclear AO green fluorescence. D. Dense cytoplasmic green fluorescence and few red labeled lysosomes in cells exposed for 24 h to 40 μM MCLR. E. Cells treated with 5 μM MCLR for 48 h with low density of cytoplasmic lysosomes. F. Control cells showing a reticular network of mitochondria. G. After exposure to 5 μM MCLR/24 h cells appear similar to the negative control. H. Cells treated with 20 μM MCLR for 24 h showing some mitochondria with altered shape. I. Note the change of the reticular pattern of mitochondria and the increase on diffuse labeling in cells treated with 40 μM MCLR for 24 h. J. Cells treated with 5 μM for 48 h, with a diffuse labeling or presenting spherical mitochondria. Scale bar 10 μm.

Please cite this article in press as: Alverca, E., et al., Morphological and ultrastructural effects of microcystin-LR from *Microcystis aeruginosa* extract on a kidney cell line, Toxicon (2009), doi:10.1016/j.toxicon.2009.04.014

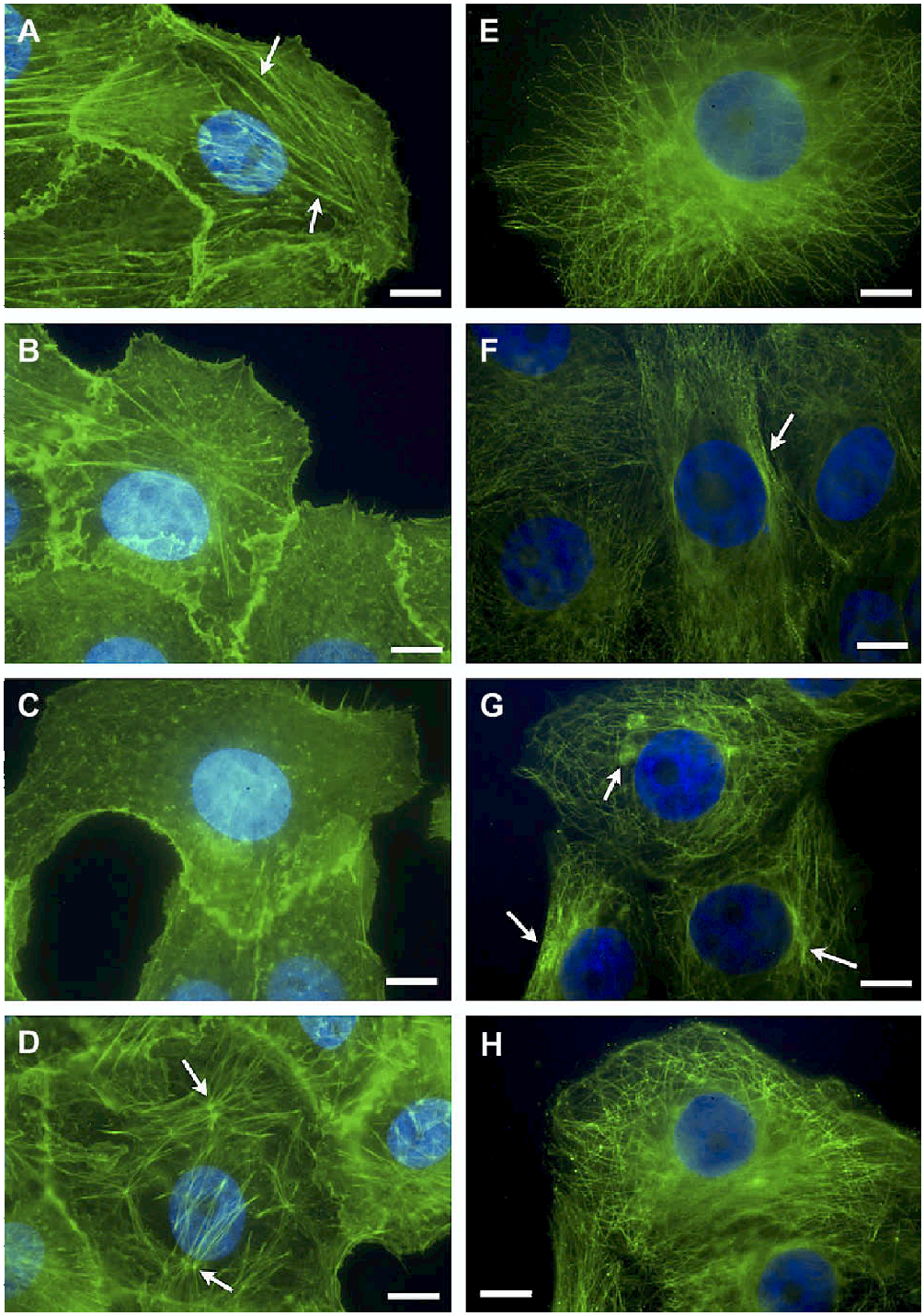


Fig. 4. Fluorescence microscopy analysis of Vero cells microfilaments stained with Alexa Fluor® 488 Ph (A–D) or microtubule immunolabelling (E–H) after treatments with MCLR. A. Control cells showing a highly organized actin mesh with abundant stress fibers (arrows). B. Cells treated for 24 h with 20 μM MCLR with reduced number of stress fibers. C. Note a drastic microfilaments disassembly after exposure to 40 μM/24 h. D. Cells treated with 5 μM for 48 h showing the partial collapse of actin fibers into cytoplasmic points (arrows). E. Control cells with a dense network of microtubules radiating from the nucleus. F and G. Cells treated for 24 h with 20 μM and 40 μM MCLR respectively, showing the intense fluorescence beams in the nucleus periphery (arrows). H. Cells exposed to 5 μM MCLR for 48 h, showing already a slight microtubules aggregation. Scale bar 10 μm.

endoplasmic reticulum stress (ERS) response is tightly linked to the mechanisms of autophagy activation, and that these two systems have interdependent controls, even though they function independently (Schönthal, 2008). The co-occurrence of ER ultrastructural changes and morphological autophagy markers in MCLR treated Vero cells is consistent with this knowledge, and suggests that the ER participates in an early MCLR-induced autophagic response.

At toxin concentrations higher than 10 μM, there were a growing number of cells presenting marked alterations on several subcellular structures, namely membrane

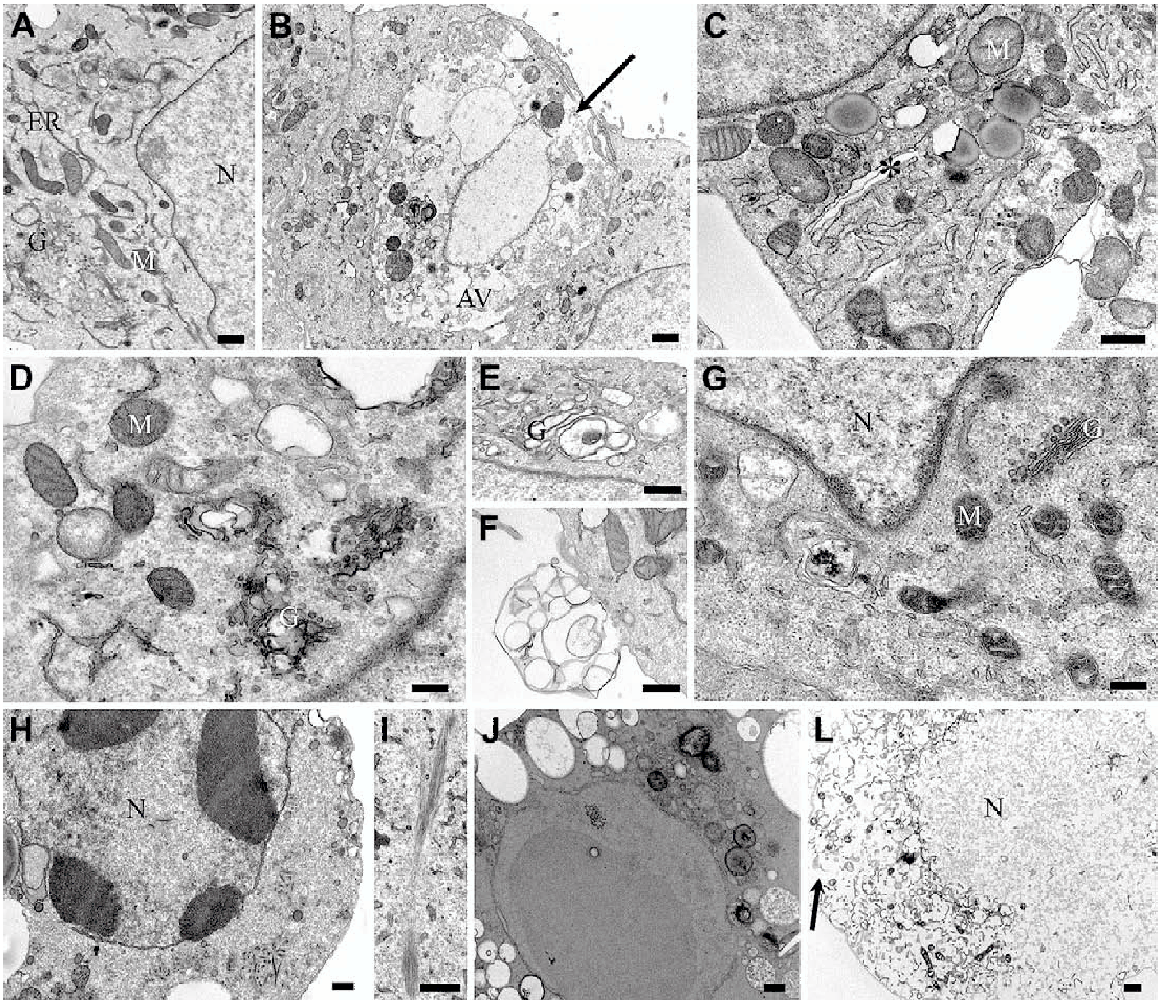


Fig. 5. Transmission electron micrographs of Vero cells exposed to different MCLR treatments A. Control cells; B and C. 5 μM MCLR for 24 h, cells showing a large autophagic vacuole (arrow) (B) and swelling of the endoplasmic reticulum (*) (C). D. 5 μM MCLR for 48 h, cell with vacuolated Golgi apparatus. E and F. 10 μM MCLR for 24 h, cells with clear vacuolization of the Golgi (E) and blebbing of the cell membrane (F). G. 10 μM MCLR for 48 h, cell showing small and condensed mitochondria. H and I. 20 μM MCLR for 48 h, cells showing chromatin condensation (H) and cytoplasmic aggregation of microtubules forming a bundle (I). J. 30 μM MCLR for 48 h, apoptotic cell with vacuolated cytoplasm and condensed nucleus. L. 75 μM MCLR for 48 h, necrotic cell with disrupted membranes (arrow). (N) Nucleus; (M) Mitochondria; (AV) Autophagic Vacuole; (ER) Endoplasmic Reticulum; (G) Golgi apparatus. Scale bar 0.5 μm.

blebbing and chromatin condensation. Similar effects were also observed by other authors in primary and permanent cell lines exposed to pure MCLR or cyanobacterial extracts (Li et al., 2001; Mankiewicz et al., 2001; McDermott et al., 1998), which were associated with apoptosis. Accordingly, our TUNEL results showed an increase of apoptosis in cells exposed to the toxin, in a dose- and time-dependent manner.

The exact mechanisms involved in MCLR-induced apoptosis are still unknown. However, there is growing evidence that the increase of ROS generation, the onset of mitochondrial permeability transition (MPT) and the loss of mitochondrial membrane potential (MMP) have fundamental roles in this process (for a review see Ding and Ong 2003). *In vivo* studies developed in mouse liver suggested that MCLR stimulates ROS formation, and related this increase with a higher expression of pro-apoptotic members of Bcl-2 family (Weng et al., 2007), supporting the importance of oxidative stress on MCLR-induced apoptosis. Besides its association with mitochondria, Bcl-2 proteins are also bound to the ER, and they appear to be involved in an alternative pathway of mitochondrial apoptosis activation (Nutt et al., 2002; Pinton et al., 2001). Apparently this process is regulated by Bcl-2 proteins localized at the ER and not at mitochondria (Scorrano et al., 2003). Additionally, it has been shown that in yeast the ER oxidative stress activates autophagy, and when autophagy is blocked the ERS-induced cell death is enhanced (Ogata et al., 2006; Yorimitsu et al., 2006). Our ultrastructural observations are consistent with these data, supporting the idea that changes in the ER can trigger both autophagic and apoptotic cellular response to MCLR.

As previously mentioned, in our experiments mitochondrial alterations were always preceded by MCLR-induced lysosomal damages. The importance of lysosome disruption as an inductor of mitochondrial membrane destabilization has already been suggested by several authors (for a review see Kurz et al., 2008). Additionally, it has been demonstrated that the partial release of lysosomal enzymes into the cytosol may induce apoptosis either directly by pro-caspase activation and/or indirectly, by mitochondrial attack and consequent release of

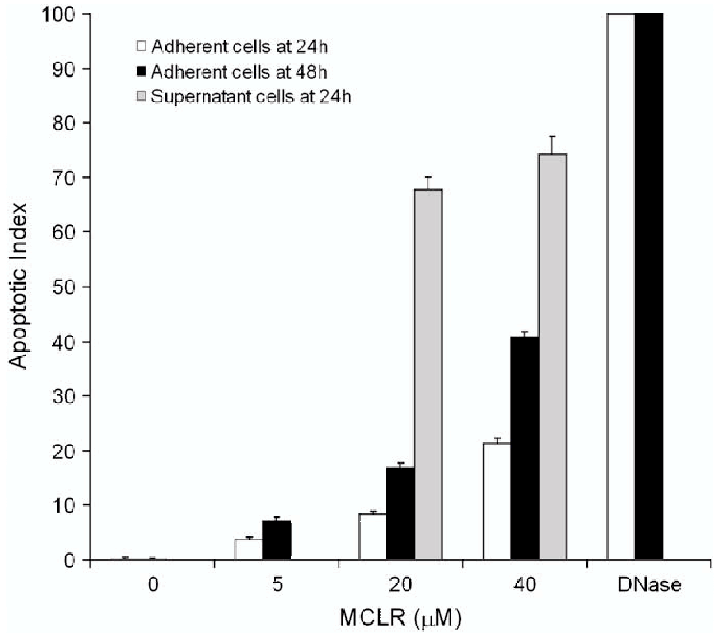


Fig. 6. MCLR-induced apoptosis in Vero cells, analyzed by the TUNEL assay. The apoptotic index represents the percentage of apoptotic cells within a total of 500 scored cells. The effects were evaluated in adherent cells after 24 h and 48 h of exposure to 5, 20 and 40 μM of MCLR. The apoptotic index of supernatant cells was determined after 24 h of exposure to 20 and 40 μM of MCLR. DNase was used as positive control. The results are expressed as means ± SD from two independent experiments.

cytochrome *c* (Li et al., 2000). These data suggest that in Vero cells there is a lysosome–mitochondria crosstalk which is important for the MCLR-induced apoptosis activation and that lysosomal permeabilization is an early event in the apoptotic cascade.

In the present study, an increase in the number of cells presenting signs of necrosis and an extensive rupture of the lysosomal apparatus became evident after prolonged exposure to high concentrations of MCLR (75 μM/48 h). Studies using the lysosomotropic detergent MSDH indicated that the key factor in determining the type of cell death is the magnitude of lysosomal permeabilization and the amount of proteolytic enzymes released into the cytosol (Li et al., 2000). Thus, it seems that in Vero cells partial, selective lysosome permeabilization triggers apoptosis, whereas massive breakdown of lysosomes results in necrosis.

MCLR is a potent inhibitor of phosphatases PP1 and PP2A, important to the cytoskeleton integrity. The disassembly of cytoskeleton induces the loss of cell contact, detachment from the growth surface and severe morphological changes (Li et al., 2001). The MCLR effects on cytoskeleton components have been extensively described (Batista et al., 2003; Frangež et al., 2003; Toivola and Eriksson, 1999; Li et al., 2001; Ito et al., 1997; Khan et al., 1996, 1995). In general, these effects are characterized by the aggregation or collapse of both MTs and MFs towards the interior of the cells and in some cases the loss of cytoskeleton structure (Ding et al., 2000b; Khan et al., 1996). The MF and MT alterations reported here are in accordance with these authors. However, the cytoskeleton disorganization was only evident at toxin concentrations and exposure times higher than those affecting other subcellular compartments. In several eukaryotic cells, it has been reported that autophagy requires a functional cytoskeleton network for its initiation and progression, thus appearing largely preserved during that process, whereas during apoptosis it is disassembled early in the process (Bursch et al., 2000). According to this, our findings further support the hypothesis that MCLR induces either an autophagic or an apoptotic cellular response depending on the exposure conditions.

Our results indicate that Vero cells respond to MCLR-induced stress through autophagy, apoptosis or ultimately through necrosis. The type of response seems to be highly dependent on the strength of the stimulus. Furthermore, the data presented also suggest that MCLR-mediated autophagy and apoptosis involve a considerable crosstalk among several organelles, in which the ER seems to have a determinant role.

It has been described that the ER stress is implicated in several kidney diseases (Pallet et al., in press) and in the nephrotoxicity of several compounds (Cribb et al., 2005). Moreover, increasing evidences suggest that the kidney might be a target organ for microcystins (Nobre et al., 1999; Milutinović et al., 2003; Gaudin et al., 2008). Additionally, a microcystin carrier (OATP-A) has already been identified at mRNA level in the human kidney (Hagenbuch and Meier, 2003). Thus, further studies conducted with distinct kidney cell models should elucidate if microcystins are nephrotoxic and, in that case, if RE-targeting is a general mechanism for the microcystins-mediated nephrotoxicity.

Acknowledgements

To Portuguese Foundation for Science and Technology for grant SFRH/BD/10585/2002 attributed to Elsa Dias. To National Health Institute, Portugal, for a master fellowship attributed to Mariana Andrade. To Institute for Medicine and Pharmaceutical Sciences (I-Med) and to Centre for Research on Human Molecular Genetics (CIGMH).

Conflicts of interest

The authors declare that there are no conflicts of interest.

References

Batista, T., De Sousa, G., Suput, J.S., Rahmani, R., Šuput, D., 2003. Microcystin-LR causes the collapse of actin filaments in primary human hepatocytes. Aquat. Toxicol. 65, 85–91.

Bauvy, C., Gane, P., Arico, S., Codogno, P., Ogier-Denis, E., 2001. Autophagy delays sulindac sílfide-induced apoptosis in the human intestinal colon cancer cell line HT-29. Exp. Cell Res. 268, 139–149.

Berg, K., Wyman, J., Carmichael, W., Dabholkar, A., 1988. Isolated rat liver perfusion studies with cyclic heptapeptide toxins of *Microcystins* and *Oscillatoria* (freshwater cyanobacteria). Toxicon 26, 827–837.

Billam, M., Mukhi, S., Tang, L., Weimin, G., Wang, J.-S., 2008. Toxic response indicators of microcystin-LR in F344 rats following a single dose treatment. Toxicon 51, 1068–1080.

Bischoff, K., 2001. The toxicology of microcystin-LR: occurrence, toxicokinetics, toxicodynamics, diagnosis and treatment. Vet. Hum. Toxicol. 43, 294–297.

Boaru, D.A., Dragoş, N., Schrimer, K., 2006. Microcystin-LR induced cellular effects in mammalian and fish primary hepatocyte cultures cell lines: a comparative study. Toxicology 218, 134–148.

Borenfreund, E., Puerner, J., 1985. Toxicity determined in vitro by morphological alterations and neutral red absorption. Toxicol. Lett. 24, 119–124.

Botha, N., Gehringer, M.M., Downing, T.G., Venter, M., Shepard, E.G., 2004. The role of microcystin-LR induction of apoptosis and oxidative stress in CaCo2 cells. Toxicon 43, 185–192.

Bouaïcha, N., Maatouk, I., 2004. Microcystin-LR and nodularin induce intracellular glutathione alteration, reactive oxygen species production and lipid peroxidation in primary cultured hepatocytes. Toxicol. Lett. 148, 53–63.

Bröker, L.E., Kruyt, F.A.E., Giaccone, G., 2005. Cell death independent of caspases: a review. Clin. Cancer Res. 11, 3155–3162.

Bursch, W., Hochegger, K., Török, L., Marian, B., Ellinger, A., Hermann, R.S., 2000. Autophagic and apoptotic types of programmed cell death exhibit different fates of cytoskeletal filaments. J. Cell. Sci. 113, 1189–1198.

Carmichael, W.W., 1994. The toxins of cyanobacteria. Nat. Toxins 270, 64–72.

Chen, T., Qingsong, W., Cui, J., Yang, W., Shi, Q., Hua, Z., Ji, J., Shen, P., 2005. Induction of apoptosis in mouse liver by microcystin-LR. Mol. Cell. Proteomics 4, 958–974.

Chorus, I., Falconer, I.R., Salas, H.J., Bartram, J., 2000. Health risks caused by freshwater cyanobacteria in recreational waters. J. Toxicol. Environ. Health B Crit. Rev. 3, 323–347.

Cribb, A.E., Peyrou, M., Muruganandan, S., Schneider, L., 2005. The endoplasmic reticulum in xenobiotic toxicity. Drug Metab. Rev. 37, 405–442.

Dabholkar, A.S., Carmichael, W.W., 1987. Ultrastructural changes in the mouse liver induced by hepatotoxin from the freshwater cyanobacterium *Microcystis aeruginosa* strain 7820. Toxicon 25, 285–292.

Davidson, H.W., McGowan, C.H., Balch, W.E., 1992. Evidence for the regulation of exocytic transport by protein phosphorylation. Trends Cell Biol. 2, 156–168.

Dias, E., Andrade, M., Alverca, E., Pereira, P., Batoréu, M.C.C., Jordan, P., Silva, M.J., 2009. Comparative study of the cytotoxic effect of microcystin-LR and purified extracts from *M. aeruginosa* on a kidney cell line. Toxicon. doi:10.1016/j.toxicon.2009.01.029.

Dias, E., Pereira, P., Batoréu, M.C.C., Jordan, P., Silva, M.J., et al., 2008. Cytotoxic and genotoxic effects of microcystins in mammalian cell lines. In: Moestrup, Ø. (Ed.), Proceedings of the 12th International Conference on Harmful Algae. ISSHA and IOC – UNESCO, Paris, pp. 282–285.

Ding, W.-X., Ong, C.-N., 2003. Role of oxidative stress and mitochondrial changes in cyanobacteria-induced apoptosis and hepatotoxicity. FEMS Microbiol. Lett. 220, 1–7.

Ding, W.-X., Shen, H.-M., Ong, C.-N., 2001. Pivotal role of mitochondrial Ca^{2+} in microcystin-induced mitochondrial permeability transition in rat hepatocytes. Biochem. Biophys. Res. Commun. 285, 1155–1161.

Ding, W.-X., Shen, H.-M., Ong, C.-N., 2000a. Critical role of reactive oxygen species and mitochondrial permeability transition in microcystin-induces rapid apoptosis in rat hepatocytes. Hepatology 32, 547–555.

Ding, W.-X., Shen, H.-M., Ong, C.-N., 2000b. Microcystic cyanobacteria extract induces cytoskeleton disruption and intracellular glutathione alteration in hepatocytes. Environ. Health Perspect. 108, 605–609.

Dinter, A., Berger, E.G., 1998. Golgi-disturbing agents. Histochem. Cell Biol. 109, 571–590.

Duy, T.N., Lam, P.K.S., Shaw, G.R., Connell, D.W., 2000. Toxicology and risk assessment of freshwater cyanobacterial (blue-green algal) toxins in water. Rev. Environ. Contam. Toxicol. 163, 113–186.

Eriksson, J.E., Toivola, D., Meriluoto, J.A.O., Karaki, H., Han, Y.-G., Hartshorne, D., 1990. Hepatocyte deformation induced by cyanobacterial toxins reflects inhibition of protein phosphatases. Biochem. Biophys. Res. Commun. 171, 1347–1353.

Fischer, W.J., Altheimer, S., Cattori, V., Meier, P.J., Dietrich, D.R., Hagenbuch, B., 2005. Organic anion transporting polypeptides expressed in liver and brain mediate uptake of microcystin. Toxicol. Appl. Pharmacol. 203, 257–263.

Fladmark, K.E., Brustugun, O.T., Mellgren, G., Krakstad, C., Bøe, R., Vintermyr, O.K., Schulman, H., Døskeland, S.O., 2002. Ca^{2+}/calmodulin-dependent protein kinase II is required for microcystin-induced apoptosis. J. Biol. Chem. 277, 2804–2811.

Fladmark, K.E., Brustugun, O.T., Hovland, R., Bøe, R., Gjertsen, B.T., Zhivotovsky, B., Døskeland, S.O., 1999. Ultrarapid caspase-3 dependent apoptosis induction by serine/threonine phosphatase inhibitors. Cell Death Differ. 6, 1099–1108.

Frangež, R., Žužek, M.C., Mrkun, J., Šuput, D., Sedmak, B., Kosec, M., 2003. Microcystin-LR affects cytoskeleton and morphology of rabbit primary whole embryo cultured cells in vitro. Toxicon 41, 999–1005.

Fu, W.Y., Chen, J.P., Wang, X.M., Xu, L.H., 2005. Altered expression of p 53, Bcl-2 and Bax induced by microcystin-LR in vivo and in vitro. Toxicon 46, 171–177.

Gaudin, J., Huet, S., Jarry, G., Fessard, V., 2008. *In vivo* DNA damage induced by the cyanotoxin microcystin-LR: comparison of intraperitoneal and oral administration by use of the comet assay. Mutat. Res. 652, 65–71.

Guzman, R.E., Solter, P.H., 1999. Hepatic oxidative stress following prolonged sublethal microcystin-LR exposure. Toxicol. Pathol. 27, 582–588.

Hagenbuch, B., Meier, P.J., 2003. The superfamily of organic anion transporting polypeptides. Biochim. Biophys. Acta 1609, 1–18.

Hicks, S.W., Machamer, C.E., 2005. Golgi structure in stress sensing and apoptosis. Biochim. Biophys. Acta 1744, 406–414.

Honkanen, E.R., Zwiller, J., Moore, E.R., Daily, S.L., Khatra, B.S., Dukelow, M., Boynton, A.L., 1990. Characterization of microcystin-LR, a potent inhibitor of type 1 and type 2A protein phosphatases. J. Biol. Chem. 265, 19401–19404.

Hooser, S.B., 2000. Fulminant hepatocyte apoptosis in vivo following microcystin-LR administration to rats. Toxicol. Pathol. 278, 726–733.

Humpage, A.R., Hardy, S.J., Moore, E.J., Froscio, S.M., Falconer, I.R., 2000. Microcystins (cyanobacterial toxins) in drinking water enhance the growth of aberrant crypt foci in the mouse colon. J. Toxicol. Environ. Health A 61, 155–165.

IARC, 2006. Cyanobacterial Peptide Toxins Available at: http://monographs.iarc.fr/ENG/Meetings/94-cyanobacteria.pdf.

Ito, E., Kondo, F., Harada, K.-I., 1997. Hepatic necrosis in aged mice by oral administration of microcystin-LR. Toxicon 35, 231–239.

Khan, S.A., Wickstrom, M.L., Haschek, W.M., Schaeffer, D.J., Ghosh, S., Beasley, V.R., 1996. Microcystin-LR and kinetics of cytoskeletal reorganization in hepatocytes, kidney cells and fibroblasts. Nat. Toxins 4, 206–214.

Khan, S.A., Ghosh, S., Wickstrom, M., Miller, L.A., Hess, R., Haschek, W.M., Beasley, V.R., 1995. Comparative pathology of microcystin-LR in cultured hepatocytes, fibroblasts and renal epithelial cells. Nat. Toxins 3, 119–128.

Kurz, T., Terman, A., Gustafsson, B., Brunk, U.T., 2008. Lysosomes and oxidative stress in aging and apoptosis. Biochim. Biophys. Acta 1780, 1291–1303.

Lankoff, A., Krzowski, Ł., Głąb, J., Banasik, A., Lisowska, H., Kuzewski, T., Góźdźm, S., Wójcik, A., 2004. DNA damage and repair in human peripheral blood lymphocytes following treatment with microcystin-LR. Mutat. Res. 559, 131–142.

Lankoff, A., Banasik, A., Obe, G., Deperas, M., Kuzminski, K., Tarczynska, M. , Jurczak, T., Wojcik, A., 2003. Effect on microcystin-LR and cyanobacterial extract from Polish reservoir of drinking water on cell cycle progression, mitotic spindle, and apoptosis in CHO-K1 cells. Toxicol. Appl. Pharmacol. 189, 204–213.

Lee, T.H., Linstedt, A.D., 1999. Osmotically induced cell volume changes alter anterograde and retrograde transport, Golgi structure, and COPI dissociation. Mol. Biol. Cell 10, 1445–1462.

Li, L., Xie, P., Chen, J., 2007. Biochemical and ultrastructural changes of the liver and kidney of the phytoplanktivorous silver carp feeding naturally on toxic *Microcystis* blooms in Taihu Lake, China. Toxicon 49, 1042–1053.

Li, L., Xie, P., Chen, J., 2005. In vivo studies on toxin accumulation in liver and ultrastructural changes in hepatocytes of the phytoplanktivorous bighead carp i.p. injected with extracted microcystins. Toxicon 46, 533–545.

Li, X.Y., Liu, Y.D., Song, L.R., 2001. Cytological alterations in isolated hepatocytes from common carp (*Cyprinus carpio* L.) exposed to microcystin-LR. Environ. Toxicol. 16, 517–522.

Li, W., Yuan, X.M., Nordgren, G., Dalen, H., Dubwchik, G.M., Firestone, R.A., Brunk, U.T., 2000. Induction of cell death by the lysosomotropic detergent MSDH. FEBS Lett. 470, 35–39.

Maag, R.S., Hicks, S.W., Machamer, C.E., 2003. Death from within: apoptosis and the secretory pathway. Curr. Opin. Cell Biol. 15, 456–461.

Mankiewicz, J., Tarczynska, M., Fladmark, K.E., Doskeland, S.O., Walter, Z., Zalewski, M., 2001. Apoptotic effect of cyanobacterial extract on rat hepatocytes and human lymphocytes. Environ. Toxicol. 16, 225–233.

Matsushima, R., Yoshizawa, S., Watanabe, M.F., Harada, K., Furusawa, M., Carmichael, W.W., Fujiki, H., 1990. *In vitro* and *in vivo* effects of protein phosphatase inhibitors, microcystins and nodularin, on mouse skin and fibroblasts. Biochem. Biophys. Res. Commun. 171, 867–874.

McDermott, C.M., Nho, C.W., Howard, W., Holton, B., 1998. The cyanobacterial toxin, microcystin-LR, can induce apoptosis in a variety of cell types. Toxicon 36, 1981–1996.

Mosmann, T., 1983. Rapid colorimetric assay for growth and survival – application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.

Milutinović, A., Živin, M., Zorc-Plesković, R., Sedmark, B., Šuput, D., 2003. Nephrotoxic effects of chronic administration of microcystins-LR and -YR. Toxicon 42, 281–288.

Nishiwaki-Matsushima, R., Otha, T., Nishiwaki, S., 1992. Liver tumor promotion by the cyanobacterial cyclic peptide toxin microcystin-LR. J. Cancer Res. Clin. Oncol. 118, 420–424.

Nobre, A.C.L., Jorge, M.C.M., Menezes, D.B., Fonteles, M.C., Monteiro, H.S.A., 1999. Effects of microcystin-LR in isolated perfused rat kidney. Braz. J. Med. Biol. Res. 32, 985–988.

Nutt, L.K., Pataer, A., Pahler, J., Fang, B., Roth, J., McConkey, D.J., Swisher, S.G., 2002. Bax and Bak promote apoptosis by modulating endoplasmic reticular and mitochondrial Ca^{2+} stores. J. Biol. Chem. 277, 9219–9225.

Ogata, M., Hino, S.-I., Saito, A., Morikawa, K., Kondo, S., Kanemoto, S., Murakami, T., Taniguchi, M., Tanii, I., Yoshinaga, K., Shiosaka, S., Hammarback, J.A., Urano, F., Imaizumi, K., 2006. Autophagy is activated for cell survival after endoplasmic reticulum stress. Mol. Cell. Biol. 26, 9220–9231.

Pallet, N., Bouvier, N., Beaunen, P., Legendre, C., Thrervet, E., Anglicheau, D. Le stress du réticulum endoplasmique au cours des néphropathies: une question de vie et de mort? Nephrol. Ther., doi:10.1016/j.nephro.2008.10.007

Philips, H.J., 1973. Dye exclusion tests for cell viability. In: Kruse, P.F., Patterson, M.K. (Eds.), Tissue Culture: Methods and Applications. Academic Press, NY, pp. 406–408.

Pichardo, S., Jos, A., Zurita, J.L., Salguero, M., Camean, A.M., Repetto, 2005. The use of the fish cell lines RTG-2 and PLHC-1 to compare the toxic effects produced by microcystins LR and RR. Toxicol. In Vitro 19, 865–873.

Pinton, P., Ferrari, D., Rapizzi, E., Di Virgilio, F.D., Pozzan, T., Rizzuto, R., 2001. The Ca^{2+} concentration of the endoplasmic reticulum is a key determinant of ceramide-induced apoptosis: significance for the molecular mechanism of Bcl-2 action. EMBO J. 20, 2690–2701.

Ramanan, S., Tang, J., Velayudhan, A., 2000. Isolation and preparative purification of microcystin variants. J. Chromatogr. A. 883, 103–112.

Rao, P.V.L., Bhattacharya, R., Parida, M.M., Jana, A.M., Bhaskar, A.S.B., 1998. Freshwater cyanobacterium *Microcystis aeruginosa* (UTEX 2385) induced DNA damage *in vivo* and *in vitro*. Environ. Toxicol. Pharmacol. 5, 1–6.

Schönthal, A.H., 1999. Endoplasmic reticulum stress and autophagy as targets for cancer therapy. Cancer Lett. 275, 163–169.

Scorrano, L., Oakes, S.A., Opferman, J.T., Cheng, E.H., Sorcinelli, M.D., Pozzan, T., Korsmeyer, S.J., 2003. BAX and BAK regulation of endoplasmic reticulum Ca^{2+}: a control point for apoptosis. Science 300, 135–139.

Skulberg, R., Skulberg, O.M., 1990. Forskning med algekulturer NIVAs kultursampling av alger. NIVA, Norway, 32 pp.

Toivola, D.M., Eriksson, J.E., 1999. Toxins affecting cell signaling and alteration of cytoskeletal structure. Toxicol. In Vitro 13, 521–530.

Ueno, Y., Nagata, S., Tsutsumi, T., Hasegawa, A., Watanabe, M.F., Park, H.D., Chen, G.C., Chen, G., Yu, S.Z., 1996. Detection of microcystins, a blue-green algal hepatotoxins, in drinking water sampled in Haimen and Fusui, endemic areas of primary liver cancer in China, by highly sensitive immunoassay. Carcinogenesis 17, 1317–1321.

Valério, E., Chambel, L., Paulino, S., Faria, N., Pereira, P., Tenreiro, R., 2009. Multiplex PCR for detection of microcystins-producing cyanobacteria from freshwater samples. Environ. Toxicol., in press.

Weng, D., Lu, Y., Wei, Y., Liu, Y., Shen, P., 2007. The role of ROS in microcystin-LR-induced hepatocytes apoptosis and liver injury in mice. Toxicology 232, 15–23.

Yorimitsu, T., Nair, U., Yang, Z., Klionsky, D.J., 2006. Endoplasmic reticulum triggers autophagy 40, 30299–30304.

Zhao, Y., Xie, P., Tang, R., Zhang, X., Li, L., Li, D., 2008. *In vivo* studies on the toxic effects of microcystins on mitochondrial electron transport chain and ion regulation in liver and heart of rabbit. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 148, 204–210.

Please cite this article in press as: Alverca, E., et al., Morphological and ultrastructural effects of microcystin-LR from *Microcystis aeruginosa* extract on a kidney cell line, Toxicon (2009), doi:10.1016/j.toxicon.2009.04.014

CAPÍTULO 5

Dias, E., Santos, T., Pereira, P., Batoréu, M.C.C., Jordan, P., Silva, M.J., Genotoxicity of microcystin-LR on Vero-E6 and HepG2 cell lines evaluated by the Comet and the Micronucleus assays (em preparação para submissão).

Genotoxicity of microcystin-LR on Vero-E6 and HepG2 cell lines evaluated by the Comet and the Micronucleus assays.

Authors:

Elsa Dias[1,2,3*], Telma Santos[1], Paulo Pereira[2], M.C.C. Batoréu[3], Peter Jordan[1], Maria João Silva[1]

Affiliations:

[1] Department of Genetics, National Institute of Health Dr. Ricardo Jorge, Av. Padre Cruz, 1649-016 Lisbon, Portugal

[2] Department of Environmental Health, National Institute of Health Dr. Ricardo Jorge, Av. Padre Cruz, 1649-016 Lisbon, Portugal

[3] University of Lisbon, Faculty of Pharmacy, I-Med, Laboratory of Toxicology, Av. Prof. Gama Pinto, 1649-019 Lisbon, Portugal

* Corresponding author - phone: + 351 217519393, fax: + 351 217526400, email: elsa.dias@insa.min-saude.pt

Abstract

Microcystins are a class of potent hepatotoxins produced by freshwater cyanobacteria, which constitute a risk for human health. Despite their well known acute toxic effects in liver, chronic toxicity has not been sufficiently studied and constitutes an important issue to be clarified for risk assessment of human exposure. It is recognized that microcystins are potentially carcinogenic through their tumour promoter activity and some evidences exist that they might also display genotoxic activity. However, uncertainties about the mechanisms involved in their potential genotoxicity and carcinogenicity still remain. Furthermore, it has been demonstrated that they may target organs other than liver, such as kidney, producing acute and chronic nephrotoxicity. In this work, we used the alkaline version of the single cell gel electrophoresis (comet assay) and the micronucleus (MN) assay in cytokinesis-blocked cells to characterize the genotoxicity of microcystin-LR (MCLR), the most toxic and widespread variant of microcystins, in a monkey kidney (Vero-E6) and a human hepatoma-derived (HepG2) cell lines. The results show that non-cytotoxic concentrations of MCLR induce a significant increase in the frequency of MN in both cell lines, although it does not produce a detectable level of DNA damage in the comet assay. These findings are in agreement with previous MN data obtained with an extract from a MCLR-*M. aeruginosa* producer strain in Vero cells and indicate that MCLR is able to produce a genotoxic effect in cell lines derived from liver and kidney, which might be related to a clastogenic or to an aneugenic effect.

Keywords: Microcystin-LR, genotoxicity, micronuclei, comet assay, Vero-E6, HepG2 cells.

Introduction

The natural and anthropogenic-induced eutrophication of water resources has contributed to the worldwide increase in massive proliferation (bloom) of cyanobacteria in surface freshwater reservoirs. This phenomenon constitutes a risk for public health, given the ability of cyanobacteria to produce toxic secondary metabolites, including hepatotoxins (microcystins, nodularin, cylindrospermopsin), neurotoxins (saxitoxin and derivatives, anatoxins) and lipopolysaccharides (revised in Duy et al, 2000). Microcystins (cyclic heptapeptides) are widespread hepatotoxins produced by several cyanobacterial species, such as *Microcystis aeruginosa,* and have been associated with episodes of human and animal acute liver toxicity (Chorus et al., 2000; Duy et al., 2000).

The liver specificity of microcystins has been attributed to the selective expression of a family of membrane transporters, the Organic Anion Polypeptide Transporter (OAPT), by the hepatocytes (Hagenbuch and Meier, 2003, Fisher et al., 2005), through which microcystins enter into the cells. The mechanism of acute hepatotoxicity is believed to be mediated by the inhibition of serine/threonine protein phosphatases 1 and 2A (PP1 and PP2A) in the liver cells (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, necrosis, liver haemorrhage and death (Falconer and Yeung, 1992). Increasing evidences have demonstrated that microcystins also might target other organs such as the kidney (Nobre et al., 1999; Milutinović et al., 2002, 2003), intestine (Botha et al, 2004; Gaudin et al, 2008), lungs (Soares et al., 2007), reproductive system (Ding et al, 2006) and brain (Maidana et al., 2006), although the knowledge of the toxicokinetics and toxicodynamics of microcystins on these organs is still very restricted.

In 1998, the World Health Organization (WHO, 1998) defined a drinking water guideline value of 1.0 $\mu g.L^{-1}$ (1 nM) for microcystin-LR (MCLR), the most toxic and prevalent microcystin variant. This value was calculated from the acute hepatotoxicity data in experimental animals and, thus, it only accounts for the prevention of acute liver hazards. Although the adverse effects from repeated and prolonged exposure to low doses of MCLR through drinking water have not been taken into consideration, they might also be relevant for the assessment of risks to the human health. Epidemiological studies have suggested an association between human chronic exposure to low doses of

microcystins in drinking water and an increase in primary hepatocellular (Yu, 1995; Ueno et al., 1996) and colorectal cancers (Zhou et al, 2002). Moreover, two-stage rodent carcinogenesis assays have demonstrated that MCLR is involved in tumour promotion in rat liver (Nishiwaki-Matsushima et al. 1992) and mouse skin (Falconer, 1991) and tumour progression in mouse colon (Humpage et al., 2000a). It has been proposed that the tumour-promoting activity of microcystins is mediated by its inhibitory effect on protein phosphatases PP1 and PP2A, resulting in the activation of the proto-oncogenes *c-jun*, *c-fos* and *c-myc* (Gehringer, 2004; Li et al., 2009).

Additionally, it has been equally suggested that MCLR might be a complete carcinogen because it is able to induce *per se* the formation of neoplastic nodules in mouse liver, without pre-treatment with a tumour initiator (Ito et al., 1997). Data from several *in vivo* and *in vitro* genotoxicity studies have been corroborating this finding, showing that MCLR might have genotoxic activity, although this is still a matter of some controversy. Another possibility is that MCLR interferes with DNA repair processes, namely the repair of gamma radiation- and ultra-violet-induced DNA damage (Lankoff et al., 2004; 2006a,b), thus increasing the genotoxic potency of those agents and contributing to their carcinogenesis.

The results from mutagenesis assays have not been conclusive. The early papers by Grabow et al. (1982) and Repavich et al. (1990) reported that extracts from *Microcystis* sp. failed to induce point mutations in the Ames test. In line with these data, Wu et al. (2006) did not find any mutagenic activity of cyanobacterial extracts in several short-term assays (ara, Ames and SOS/umu assays). Conversely, in another study, a *Microcystis aeruginosa* extract yielded a positive result in the Ames test, while pure MCLR gave a negative result (Ding et al., 1999), such as previously shown by Tsuji et al. (1995). On the other hand, results from mutation analyses in endogenous genes of human cells showed that MCLR is able to increase the frequency of ouabain-resistant RSa mutant cells (Suzuki et al., 1998) as well as the frequency of mutations in the *hprt* gene of the TK6 lymphoblastoid cell line (Zhan et al., 2004).

Several reports have shown that MCLR induces DNA damage in liver cells *in vivo* (Rao and Bhattacharya, 1996; Rao et al., 1998; Gaudin et al, 2008), in cultured hepatocytes (Ding et al., 1999; Žegura et al, 2003, 2004, 2006; Nong et al., 2007) and in other cell types (Rao et al., 1998; Mankiewicz et al., 2002; Lankoff et al., 2004; Žegura et al, 2008a). However, the mechanism behind the observed DNA breakage is not clear and seems to be dose- and cell type- dependent. A study with rat liver cells has shown

that MCLR does not induce the formation of MCLR-DNA adducts, which would represent a pre-mutagenic lesion (Bouaïcha et al., 2005) and, to our knowledge, no MCLR adduct has been identified so far, suggesting an indirect mechanism for its genotoxicity. In fact, some authors have attributed the MCLR-induced DNA lesions measured in the comet assay to endonucleolytic DNA degradation associated with apoptosis (Lankoff et al., 2004) or necrosis (Rao et al., 1998) rather than to genotoxic events. In contrast, some studies have shown that the positive results produced by subcytotoxic concentrations of MCLR in the comet assay, using the human hepatoma HepG2 (Žegura *et al*. 2003, 2004, 2006; Nong et al., 2007) and colon carcinoma CaCo2 (Žegura *et al* 2008a) cell lines, were a consequence of oxidative stress and, thus, of an indirect genotoxic effect. Supporting this hypothesis, studies with liver cells have demonstrated that subcytotoxic doses of MCLR induce the formation of 8-oxo-dG, a marker of oxidative DNA damage (Maatouk et al., 2004; Bouaïcha et al., 2005). In addition, reactive oxygen species (ROS) are believed to play a central role in the MCLR-mediated apoptosis (Revised in Ding and Ong, 2003).

A chromosome damaging activity has been suggested for microcystins by an increase of the micronucleus (MN) frequency in mouse erythrocytes (Ding et al., 1999) and in the human TK6 cell line (Zhan et al., 2004). However, no effect on the micronucleus frequency has also been reported for MCLR-treated CHO-K1 cells (Fessard et al., 2004; Lankoff et al., 2006a) in agreement with the negative results of the chromosome aberrations analysis in lymphocytes (Lankoff et al., 2004, 2006b).

In previous reports, we showed that a monkey kidney (Vero-E6) cell line presented a higher sensitivity to the cytotoxic effects of an extract from a MCLR-producer *Microcystis aeruginosa* strain, than human (HepG2) and mouse (AML12) hepatocyte cell lines. Additionally, we observed that the same extract induced a significantly increased frequency of MN in Vero cells (Dias et al., 2008). However, we could not attribute undoubtedly those effects to MCLR because cyanobacterial extracts may contain other bioactive compounds that could interfere with overall extracts toxicity (Falconer, 2007). In further studies, we confirmed that pure (commercial) MCLR triggers a dose- and time-dependent cytotoxic effect on Vero cells (Dias et al., 2009). Depending on the toxin concentration, those effects included morphologic and ultrastructural alterations of cellular organelles consistent with autophagy, apoptosis or necrosis (Alverca et al., 2009). According to those previous results, we considered that

Vero cells constitute a valuable cell model to study MCLR toxicity, particularly, genotoxicity.

The aim of the present work was to characterize the genotoxic effects of pure MCLR in Vero cells, at non-cytotoxic concentrations, using the alkaline version of the comet assay and the analysis of micronucleus assay in cytokinesis-blocked cells. In addition, the same endpoints were evaluated in the liver-derived HepG2 cells, representative of the main target organ of microcystins. Our data show that MCLR does not induce DNA damage detectable by the Comet assay, but is capable of inducing micronucleation in both cell lines. We suggest that the MCLR-induced micronuclei are formed by an aneugenic mechanism.

Materials and methods

1. Microcystin-LR

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 (3 mM) was prepared by dissolving the toxin in cell culture medium. This solution was sterilized through PVDF 0.22 μm syringe filters (Millex-GV, Millipore) and kept at -20ºC until use. Work solutions of 5 and 20 μM of MCLR were prepared immediately before testing, by diluting the stock solution in cell culture medium.

2. Cell lines

The Vero-E6 (African green monkey-*Cercopithecus aethiops* kidney epithelial cells) and HepG2 (human hepatocellular carcinoma) cell lines were obtained from the American Type Culture Collection (ATCC-CRL 1586) and German Collection of Microorganisms and Cell Cultures (DSMZ ACC 180), respectively. All media and supplements were purchased from Invitrogen (Paisley, UK). Vero cells were grown in Modified Eagle Medium (MEM) supplemented with 10% FBS, 0.1mM non-essential aminoacids and 1mM sodium pyruvate. HepG2 cells were grown in RPMI 1640+Glutamax, containing 15% FBS. Both cell lines were maintained at 37ºC, in a 5% CO_2 humidified incubator.

3. Cytotoxicity (Neutral Red) assay

The NR assay (Borenfreund and Puerner, 1985) was conducted in 96-well plates containing $1x10^4$ viable cells /100µL growth medium for 24h. Cells were exposed to 5 and 20 µM of MCLR during 24 hours. H_2O_2 (400mM, 1h) was included as positive control and non-treated cells were the negative control. Mitomycin C (0.1 µg/mL, 24h) was tested as the positive control of the micronucleus assay. Three replicates were used per treatment condition. After treatment, the exposure medium was replaced by fresh culture medium containing a NR solution (50 $\mu g.mL^{-1}$) and incubated for 3h. Cells were rinsed with PBS and the incorporated NR was extracted with an ethanol: acetic acid: water (50:1:49) solution. NR incorporation was quantified spectrophotometrically at 540 nm using a Multiscan Ascent spectrophotometer (Labsystems, Helsinki, Finland). Cell viability was calculated as the percentage of ABS_{540nm} value from treated cells in relation to the negative control cells.

4. Genotoxicity assays

Comet assay

Cells were inoculated in 25 cm^2 flasks ($5x10^5$ viable cells/5 ml) for 24 hours to allow cell adherence and growth. After 24 h, the culture medium was replaced by fresh medium containing 5 and 20 µM of MCLR. Vero cells were exposed for short (2 and 4 h) and longer (24h) periods while HepG2 cells were exposed for a single 4 h period. Simultaneously, positive and negative controls (EMS 20mM, 1h and non-treated cells, respectively) were included. The comet assay was performed according to Singh et al. (1988) and Tice et al. (2000). Following exposure, $5x10^4$ cells were mixed with 80µL of 0.7% low melting point agarose to prepare 2 microgels on microscope slides previously covered with a 1% agarose layer. Cells were lysed (2.5M NaOH, 100mM Na_2EDTA, 10mM Tris-HCl, 1% N-laurylsarcosine, 10% DMSO, 1% Triton X-100, pH 10) for 1h at 4ºC and subjected to electrophoresis in alkaline conditions (300mM NaOH, 1mM Na_2EDTA, pH>13), 20 min at 25 V. Finally, slides were rinsed with neutralization buffer (0.4M Tris, 4M HCl, pH 7.5), stained with ethidium bromide (0.125 µg/µL) and analysed using a fluorescence microscope (Zeiss, Axioplan 2). Images of 50 randomly selected cells per coded slide, two slides per treatment, were analysed with image analysis software (Comet Imager 1.2.12, MetaSystems, GmbH). The % of DNA in the comet tail was selected to measure DNA damage.

Micronucleus assay

Cells were seeded in 6-well plates at a density of $5x10^4$ viable cells per well. Following 24 hours, the growth medium was replaced by fresh medium containing 5 and 20μM of MCLR and 1 hour later cytochalasin B (6 μg/mL) was added. For each experiment, negative (non-treated cells) and positive (mitomycin C, 0.1 μg/mL, 24h) controls were included. At 23h post treatment, cells were trypsinized and harvested using a hypotonic treatment (0.075M KCl; 3 min., at room temperature) followed by fixation with cold fixative (acetic acid: methanol, 1:3) and spread onto glass slides by cytocentrifugation (Shandon Cytospin, ThemoScientific, MA, USA) at 1200 rpm, 5 min. After air-dried, slide preparations were stained in 4% Giemsa.

This assay was performed in three independent experiments for Vero cells and in two independent experiments for HepG2 cells, using two replicate cultures per each treatment condition. A total of 1000 cytokinesis-blocked cells (binucleated cells) per replica was scored for the presence of micronuclei, using the criteria described by Fenech et al. (2003). In addition, the proportion of mono-, bi- and multinucleated cells was calculated by scoring 1000 cells per treatment condition. The nuclear division index (NDI) was calculated by the formula: NDI = M1+2M2+3M3+4M4)/N, where M1-M4 represents the number of cells with 1-4 nuclei and N is the total number of scored cells (Fenech et al., 2000).

5. Statistical analysis

Results are presented as mean ± SD. Statistical differences were analysed with a two-tailed Student *t*-test. $p<0.05$ was considered a statistically significant difference.

Results

We used the NR test, in parallel with the genotoxicity assays, in order to evaluate the effect of MCLR (5 and 20 μM) on cell viability. As it is demonstrated in Fig.1, none of the tested toxin concentrations induced a loss of cell viability of Vero and HepG2 cell lines.

We analysed the DNA damaging effect of pure MCLR both in Vero and HepG2 cell lines by the comet assay and data from the percentage of DNA in the tail is shown in Table I. Both MCLR concentrations (5 and 20 μM) did not increase the level of DNA lesions neither in Vero cells (2, 4 and 24 h exposure) nor in HepG2 cells (4 h exposure),

as compared to controls. Similar negative data were obtained in a single experiment using other time points (1, 6, 12 and 18h) with Vero cells (data not shown).

The genotoxicity of pure MCLR was further evaluated by the cytokinesis-blocked micronucleus (MN) assay in Vero and HepG2 cells. The results are depicted in Fig 2. In both cell lines the lowest concentration of MCLR (5 μM) was able to induce an increase in the frequency of binucleated cells with micronucleus (MNBC). In MCLR-treated HepG2 cells, the 1.8 times increase in the MNBC frequency was statistically significant as compared to the negative control (Fig. 2), while in Vero cells the observed induction in the level of MNBC did not reach statistical significance. For the highest toxin concentration tested (20 μM) a significant increase in the MNBC was found in both cell lines (1.8-fold in Vero cells and 2.1-fold in HepG2 cells) (Fig. 2). The data were also analysed in terms of induced frequency of micronucleated cells (after subtracting the level of spontaneous MNBC from that of MCLR-treated cells) to account for the different background frequencies of MNBC in the two cell lines. The frequency of MNBC induced by 20 μM of MCLR was 17 and 20 per 1000 cytokinesis-blocked Vero and HepG2 cells, respectively (Fig. 3).

For each treatment condition the proportion of mono, bi- and multinucleated cells was calculated per 1000 cells to evaluate potential effects of MCLR on cell cycle progression. The percentage of binucleated cells varied between 45%-56% and 64%-72% in Vero and HepG2 cultures, respectively, and no differences were observed between MCLR-treated and non-treated cells, independently of the dose. The percentages of binucleated cells are in agreement with values for optimal culture conditions (Fenech, 2000; Parry et al., 2002). The estimated NDI was 1.5-1.6 for Vero cells and 1.6-1.7 for HepG2 cells, and was not affected by the MCLR treatment. This suggests that MCLR does not interfere with the normal progression of Vero and HepG2 through the cell cycle and confirms that the tested toxin concentrations do not impact on cell proliferation (Rosefort et al., 2004).

Discussion

The increasing use of surface freshwater reservoirs as a source of drinking water is raising the problem of prolonged human exposure to cyanobacterial toxins, particularly microcystins. The establishment of the WHO guideline for microcystins constitutes a preventive measure to human health but, given that it only accounts for the acute hepatotoxic effects, the foreseeable prevention can only be partial. MCLR is

classified by IARC (2006) as a 2B class compound, that is, a potential carcinogen to humans. However, the scientific knowledge is still insufficient to clearly ascertain a complete toxicological profile of MCLR, particularly regarding its carcinogenic potential.

In the present work, we combined the comet and the MN assays to characterize the potential genotoxicity of MCLR and its mechanisms on Vero and HepG2 cell lines, since in the comet assay primary and potentially reversible DNA lesions are detected, while MN represent irreversible chromosome damage, either chromosome breakage or chromosome loss (Valentin-Severin et al., 2003). The human hepatocarcinoma HepG2 cell line has been considered a suitable cell model to evaluate the genotoxicity of many compounds because these cells retain the activity of metabolizing/detoxifying enzymes as well as many of the morphologic characteristics of liver cells (Valentin-Severin et al., 2003; Knasmüller et al., 2004a). This is particularly important for MCLR, since liver is its primary target organ. Likewise, the Vero cell line has been used for mutagenicity research (Chen et al., 2009) and, in preceding works, we showed that this cell line constitutes a valuable *in vitro* model to evaluate the nephrotoxicity of MCLR (Alverca et al., 2009; Dias et al., 2009). Our previous results also revealed that a *M. aeruginosa* extract containing 20 μM of MCLR induced a 3.4-fold increase in the frequency of micronucleated Vero cells (Dias et al., 2008). The results were in agreement with those of Ding et al. (1999), who found a 5-fold increase in bone marrow micronucleated polychromatic erythrocytes from mice injected (i.p.) with a toxic extract from a *M. aeruginosa* strain (45 μg MCLR / Kg bw), although those positive results could not be unequivocally attributed to MCLR, since cyanobacterial extracts could contain other bioactive compounds contributing to the overall extract toxicity (Falconer, 2007).

In the present work, we show that pure MCLR at non-cytotoxic concentrations (5-20 μM) is able to increase the frequency of micronucleus, both in Vero and in the human HepG2 cells. A similar effect has been previously described in the human lymphoblastoid TK6 cell line, treated for 24 h with 40 μM of MCLR (Zhan et al., 2004). In contrast, studies using the Chinese hamster ovary (CHO-K1) cell line exposed to either MCLR (5 μM) or a purified extract from *M. aeruginosa* (containing 1, 10 and 20 μM of MCLR) failed to detect a significant induction of micronuclei (Fessard et al., 2004; Lankoff et al., 2006a). Micronuclei can reflect events of both chromosome breakage and chromosome loss and thus an increase in the frequency of micronuclei can be related to clastogenic and/or aneugenic effects. Despite inducing

micronuclei, MCLR is not able to induce neither chromosome aberrations in lymphocytes (Lankoff, 2004, 2006b) nor sister chromatid exchanges in Vero cells exposed to a purified extract from a toxic *M. aeruginosa* containing up to 40 μM of MCLR (unpublished data), indicating that it is not clastogenic and suggesting that it may rather act as an aneugenic agent. To our knowledge, however, the nature of MCLR-induced micronuclei has not been determined, yet.

Several experimental evidences have been pointing to the possibility of an indirect disturbance of the mitotic spindle by MCLR, through its well documented inhibitory activity of protein phosphatases PP1/PP2A (Honkanen et al., 1990; Matsushima et al., 1990). The effect of MCLR on cytoskeleton components is, indeed, well known (Khan et al., 1995, 1996; Toivola and Eriksson, 1999; Batista et al., 2003; Billam et al., 2008) and we also have observed MCLR-induced morphological and ultrastructural changes (disassembly, depolimerization, aggregation) in microfilaments and microtubules of Vero cells (Alverca et al., 2009) at the same dose range (5 - 20 μM) as that reported herein to cause micronucleus formation in Vero and HepG2 cell lines. In addition, it is known that protein phosphatases PP1 and PP2A, through their central role on phosphorylation-dephosphorylation reactions, participate in the control of assembly and constant turnover of microtubules (Tournebize et al., 1997), required for both spindle formation and chromosome movement (Cassimeris, 1999). In fact, it was demonstrated for CHO-K1 cells that MCLR (≥ 50 μM) induces damages in mitotic spindle apparatus (Lankoff et al., 2003). Thus, it can be hypothesized that the observed MCLR-induced micronucleation would be mediated by an aneugenic activity. Using FISH with a human pancentromeric DNA probe other authors demonstrated that nodularin - a MCLR-structurally related cyanotoxin which also displays protein phosphatases inhibition activity - induces micronuclei that contain whole chromosomes/centric fragments in HepG2 cell line, indicative of an aneugenic activity (Lankoff et al., 2006c).

In a preliminary approach to study the MCLR genotoxic activity by the Comet assay, we tested the effect of a cyanobacterial extract from a MCLR-producer strain of *M.aeruginosa* on the Vero cell line. Several concentrations of MCLR (1, 5, 20 and 40 μM) and time-points (1, 2, 4, 6, 12, 18 and 24h) were tested in order to find a dose- and time- range for further experiments. Exposure of Vero cells to that cyanobacterial extract did not induce a detectable level of DNA damage in the alkaline version of the comet assay, for all the tested toxin concentrations and incubation periods (data not

shown). In line with that data, in the present work we show that MCLR (5 and 20 μM) does not induce DNA damage in Vero and HepG2 cell lines, independently of the time-points tested. These results disagree with previous reports of Žegura et al. (2003, 2004, 2006), describing a dose-dependent and transient induction of DNA damage by MCLR in the HepG2 cell line. The maximum level of DNA lesions was observed in HepG2 cells exposed to 1 μM of MCLR (Žegura et al., 2003, 2004) and in CaCo2 cells exposed to 5 μM of MCLR for 4 h, although other cell lines (human astrocytoma IPDDC-A2 and human B-lymphoblastoid NCNC cells) gave negative results (Žegura et al., 2008a). Nong et al. (2007) have also reported positive results in the Comet assay in HepG2 cells exposed to MCLR, but for a higher toxin dose-range (30-100 μM), raising the question of a possible confounding effect from DNA degradation due to early apoptosis, in the comet data. In another work using the comet assay, it was reported that MCLR is able to induce DNA damage in human lymphocytes in a time- and dose-dependent manner, reaching its maximum at 25 μM after 18 h exposure (Lankoff et al., 2004). However, the observed positive correlation between the frequency of apoptotic cells and the level of DNA damage led to the conclusion that the MCLR-induced DNA damage observed in the comet assay might be related to the early stages of apoptosis due to cytotoxicity and not to genotoxicity (Lankoff et al., 2004). In our work, although exposure conditions were comparable to those described above, no increase in DNA damage over control was observed. A possible explanation for this discrepancy may be that different cell lines and even distinct clones of the HepG2 cell line can generate divergent data. Knasmüller et al (2004a) reported that strong differences exist in the sensitivities of several HepG2 clones that might explain discrepant results in the comet or in the MN assays, under similar experimental conditions. For example, differences in the mechanism of MCLR uptake among different cell lines (different patterns of OATP expression or even alternative uptake mechanism) might result in different intracellular MCLR effective concentration, in cells exposed to a similar toxin dose-range. In addition, differences in the cell defences against genotoxic insults might also have several outcomes in terms of DNA or chromosome damage. The mechanism proposed for MCLR-induced DNA damage in HepG2 cells was the production of reactive oxygen species (Žegura et al., 2003, 2004, 2006; Nong et al., 2007) because it was demonstrated that MCLR stimulates the increase of intracellular glutathione content (Žegura et al., 2006) and that transient MCLR-induced DNA breaks corresponded to intermediates formed during the repair of oxidative DNA lesions (Žegura et al., 2003, 2004). In

addition, the formation of 8-oxo-dG, an oxidative DNA adduct, has been already reported in cultured primary rat hepatocytes and *in vivo* rat liver exposed to non-cytotoxic concentrations of MCLR (Maatouk et al., 2004; Bouaïcha et al., 2005). Thus, MCLR-induced DNA damage seems to depend, at least in part, from the balance between the ability of MCLR to induce oxidative stress and the cells ability to activate the cellular antioxidant defences to overcome that stress. In this way, cells more resistant to oxidative stress might not display ROS-induced DNA damage derived from MCLR treatment. The reversibility or persistence of MCLR-induced DNA lesions giving rise to mutations or chromosome alterations might depend, as well, of the DNA repair capacity of the target cells. In HepG2 cells it has been shown that MCLR transiently induces the expression of p53 and its downstream-regulated genes involved in DNA repair, providing evidence that they respond to MCLR-induced DNA damage (Žegura et al., 2008b).

In general, most compounds that give positive results in the MN assay are also positive in the comet assay and both assays seem to have identical sensitivity to detect genotoxicants (Knasmüller et al., 2004a). However, there are some exceptions, e.g., the mycotoxin citrinin, that induces MN but no DNA damage (comet assay) in HepG2 cells (Knasmüller et al., 2004b). This apparent discrepancy can be explained by the fact that citrinin induces aneuploidy, which results in MN formation but not through DNA breaks detectable by the comet test (Knasmüller et al., 2004b). Based on our data, a similar mechanism of genotoxic action through aneuploidy induction can be proposed for MCLR. However, further studies, involving characterization of the content of MCLR-induced MN with centromere specific FISH probes are needed, before a firm conclusion can be drawn.

In summary, using kidney- and liver-derived cell lines, we show that MCLR consistently induces chromosome damage, reflected as increased levels of micronuclei, although we could not observe any increase in DNA damage, using the comet assay. These data, together with those from other works on the genotoxicity of MCLR, are suggestive that MCLR might not be a clastogenic but rather an aneugenic agent. Aneuploidy induction might be a common mechanism of MCLR genotoxicity in liver and kidney cells and might result from its interference with the mitotic spindle as part of a more general effect on cell cytoskeleton. Aneuploidy has been associated with cell transformation towards malignancy and human cancer development (Kirsch-Volders et al., 2002) and thus can contribute to the carcinogenic activity of MCLR.

The distinction between clastogens and aneugens is an important feature for risk assessment because, conversely to clastogens, aneugens usually exhibit a dose-response pattern, which enables the establishment of a threshold level below which no hazard to human health is envisaged (Kirsch-Volders et al., 2002; Iarmarcovai et al., 2006). Thus, our results emphasize the importance of the revision of the provisional guideline value for MCLR taking into account the accumulating evidences that MCLR is also a carcinogenic agent, acting through genotoxic (probably aneugenic) and tumour promoting mechanisms.

Moreover, the effect of simultaneous exposure to distinct cyanotoxins should be considered for cancer risk assessment purposes because it is well known that cyanobacterial blooms are often composed by several species/strains producing different cyanotoxins (Chorus et al., 2000; Duy et al, 2000). It has been described that besides MCLR, the MCRR microcystin variant (Žegura et al., 2002) and other toxins, such as nodularin (Lankoff et al., 2006c) and cylindrospermopsin, (Humpage et al., 2000b) are also potential genotoxins. Further studies should be conducted in order to determine if the simultaneous exposure to these cyanotoxins may present an overall antagonistic or synergistic genotoxic effect.

Acknowledgements

This work was supported by the Portuguese Foundation for Science and Technology via grant SFRH/BD/10585/2002 attributed to Elsa Dias and plurianual programs for the Centre for Research on Human Molecular Genetics (CIGMH) and Institute for Medicine and Pharmaceutical Sciences (I-MED).

References

Alverca, E., et al., Morphological and ultrastructural effects of microcystin-LR from *Microcystis aeruginosa* extract on a kidney cell line, Toxicon (2009), doi: 10.1016/j.toxicon.2009.04.014.

T. Batista, G. De Sousa, J.S. Suput, R. Rahmani, D. Šuput. Microcystin-LR causes the collapse of actin filaments in primary human hepatocytes, Aquat. Toxicol. 65 (2003) 85-91.

M. Billam, S. Mukhi, L. Tang, G. Weimin, J.S. Wang. Toxic response indicators of microcystin-LR in F344 rats following a single dose treatment, Toxicon 51 (2008) 1068-1080.

E. Borenfreund, J. Puerner. Toxicity determined in vitro by morphological alterations and neutral red absorption, Toxicol. Lett. 24 (1985) 119-124.

N. Botha, M.. Van de Venter, T.G. Downing, E.G. Shepard, E.G.Gehringer. The effect of intraperitoneally administered microcystin-LR on the gastrointestinal tract of Balb/c mice, Toxicon 43 (2004) 251-254.

N. Bouaïcha, I.. Maatouk, M.J. Plessis, F. Périn.. Genotoxic potential of microcystin-LR and nodularin *in vitro* in primary cultured rat hepatocytes and *in vivo* rat liver, Environ. Toxicol. 20 (2005) 341-347.

L. Cassimeris. Accessory protein regulation of microtubule dynamics throughout the cell cycle, Current Opinion in Cell Biology 11 (1999) 134-141.

Q. Chen, S. Tang, X. Jin, J. Zou, K. Chen, T. Zhang, X. Xiao. Investigation of the genotoxicity of quinocetone, carbadox and olaquindox *in vitro* using Vero cells, Food and Chemical Toxicology 47 (2009) 328-334.

I. Chorus, I.R. Falconer, H.J. Salas, J. Bartram. Health risks caused by freshwater cyanobacteria in recreational waters, J. Toxicol. Environ. Health, Part B 3 (2000) 323-347.

E. Dias, P. Pereira, M.C.C. Batoréu, P. Jordan, M.J. Silva, M.J. Cytotoxic and genotoxic effects of microcystins in mammalian cell lines, in: Ø. Moestrup, et al. (Eds.), Proceedings of the 12th International Conference on Harmful Algae, ISSHA and IOC- UNESCO, Paris, 2008, pp. 282-285.

E. Dias, M. Andrade, E. Alverca, P. Pereira, M.C.C. Batoréu, P. Jordan, M.J. Silva. Comparative study of the cytotoxic effect of microcistin-LR and purified extracts from Microcystis aeruginosa on a kidney cell line, Toxicon 53 (2009) 487-495.

W.X. Ding, H.M. Shen, H.G. Zhu, B.L. Lee, C.N. Ong. Genotoxicity of microcistic cyanobacterial extract of a water source in China, Mutat Res 442 (1999) 69-77.

W.X. Ding, C.N. Ong. Role of oxidative stress and mitochondrial changes in cyanobacteria-induced apoptosis and hepatotoxicity, FEMS Microbio. Lett. 10880 (2003) 1-7.

X.S. Ding, X.Y. Li, H.Y. Duan, I.K. Chung, J.A. Lee. Toxic effects of Microcystis cell extracts on the reproductive system of male mice, Toxicon 48 (2006) 973-979.

T.N. Duy, P.K.S. Lam, G.R. Shaw, D.W. Connell. Toxicology and risk assessment of freshwater cyanobacterial (blue-green algal) toxins in water, Rev. Environ. Contam. Toxicol. 163 (2000) 113-186.

I.R. Falconer. Tumour promotion and liver injury caused by oral consumption of cyanobacteria, Environ. Toxicol. Water Qual. 6 (1991) 177-184.

I.R. Falconer, D.S. Yeung. Cytoskeletal changes in hepatocytes induced by microcystis toxins and their relation to hyperphosphorilation of cell proteins, Chem. Biol. Interact. 81 (1992) 181-196.

I.R. Falconer. Cyanobacterial toxins present in *Microcystis aeruginosa* extracts – More than microcystins!, Toxicon 50 (2007) 585-588.

M. Fenech, W.P. Chang, M. Kirsch-Volders, N. Holland, S. Bonassi, E. Zeiger. HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures, Mutat. Res. 534 (2003) 65–75.

M. Fenech.. The in vitro micronucleus technique, Mut. Res. 455 (2000) 81-95.

V. Fessard, L. Le Hegarat, A. Mourot. Comparison of the genotoxic results obtained from the in vitro cytokinesis-block micronucleous assay with various toxins inhibitors of protein phosphatases: okadaic acid, nodularin and microcystin-LR, in: Proceedings of Sixth International Conference on Toxic Cyanobacteria, Bergen, Norway 21-27 June, 2004, pp.68-69.

W.L. Fischer, S. Altheimer, V. Cattori, P.J. Meier, D.R. Dietrich, B. Hagenbuch.. Organic anion transporting polypeptides expressed in liver and brain mediate uptake of microcystin, Toxicol Appl Pharmacol. 203 (2005) 257-263.

J. Gaudin, S. Huet, G. Jarry, V. Fessard. *In vivo* DNA damage induced by the cyanotoxin microcystin-LR: comparison of intra-peritonial and oral administration by use of the comet assay, Mutat. Res. 652 (2008) 65-71.

M.M. Gehringer. Microcystin-LR and okadaic acid-induced cellular effects: a dualistic response, FEBS Lett. 557 (2004) 1–8.

W. Grabow, W. Randt, O. Prozesky, W. Scott. Microcystis aeruginosa toxin: cell culture toxicity, hemolysis and mutagenicity assays, Appl. Environ. Microbiol 43 (1982) 1425-1433.

B. Hagenbuch, P.J. Meier. The superfamily of organic anion transporting polypeptides, Biochimica et Biophysica Acta 1609 (2003) 1-18.

R.E. Honkanen, J. Zwiller, R.E. Moore, S.L. Daily, B.S. Khatra, M. Dukelow, A.L. Boynton. Characterization of microcystin-LR, a potent inhibitor of type 1 and type 2A protein phosphatases, J. Biol. Chem. 265 (1990) 19401-19404.

A.R. Humpage, S.J. Hardy, E.J. Moore, S.M. Froscio, I.R. Falconer. Microcystins (cyanobacterial toxins) in drinking water enhance the growth of aberrant crypt foci in the mouse colon, J. Toxicol Environm Health A 61 (2000a) 155-65.

A.R. Humpage, M. Fenech, P. Thomas, I.R. Falconer. Micronucleous induction and chromosome loss in transformed human white cells indicate clastogenic and aneugenic action of the cyanobacterial toxin, cylindrospermopsin, Mutat. Res. 472 (2000b) 155-161.

IARC. 2006. Cyanobacterial peptide toxins. Available at: http://monographs.iarc.fr/ ENG/Meetings/94-cyanobacteria.pdf.

G. Iarmarcovai, A. Botta, T. Orsière. Number of centromeric signals in micronuclei and mechansims of aneuploidy, Toxicology Letters 166 (2006) 1-10.

E. Ito, F. Kondo, K. Terao, K.I.. Harada. Neoplastic nodular formation in mouse liver induced by repeated intraperitoneal injection of microcystin-LR, Toxicon 35 (1997) 1453-1457.

S.A. Khan, S.Ghosh, M. Wickstrom, L.A. Miller, R. Hess, W.M., Haschek, V.R. Beasley. Comparative pathology of microcystin-LR in cultured hepatocytes, fibroblasts and renal epithelial cells, Nat. Toxins 3 (1995) 119-128.

S.A Khan, M.L. Wickstrom, W.M. Haschek, D.J. Schaeffer, S. Ghosh, V.R. Beasley. Microcystin-LR and kinetics of cytoskeletal reorganization in hepatocytes, kidney cells and fibroblasts, Nat. Toxins 4 (1996) 206-214.

M. Kirsch-Volders, A. Vanhauwaert, M. De Boeck, I. Decordier. Importance of detecting numerical versus structural chromosome aberrations, Mut. Res. 504 (2002) 137-148.

S. Knasmüller, V. Mersch-Sundermann, S. Kevekordes, F. Darroudi, W.W. Huber, C. Hoezl, J. Bichler, B.J. Majer. Use of human-derived liver cell lines for the detection of environmental and dietary genotoxicants; current state of knowledge, Toxicology 198 (2004a) 315-328.

S. Knasmüller, C. Cavin, A. Chakraborty, F. Darroudi, B.J. Majer, V. Ehrlich. Structurally related mycotoxins, ochratoxin A, ochratoxinB, citrinin differ in their genotoxic activities, and in their mode of action in human derived liver (HepG2) cells: implications for risk assessment, Nutr. Cancer 50 (2004b) 190-197.

A. Lankoff, A. Banasik, G. Obe, M. Deperas, K. Kuzminski, M. Tarczynska, T. Jurczak, A. Wojcik. Effect on microcystin-LR and cyanobacterial extract from Polish reservoir of drinking water on cell cycle progression, mitotic spindle, and apoptosis in CHO-K1 cells, Toxicol. Appl. Pharm. 189 (2003) 204-213.

A. Lankoff, L. Krzowski, J. Glab, A. Banasik, H. Lisowska, T. Kuszwski, S. Gozdz, A. Wojcik. DNA damage and repair in human peripheral blood lynphocytes following treatment with microcystin-LR, Mut. Res. 559 (2004) 131-142.

A. Lankoff, J. Bialczyki, D. Dziga, W.W. Carmichael, H. Lisowska, A. Wojcik. Inhibition of nucleotide excision repair by microcystin-LR in CHO-K1 cells, Toxicon 48 (2006a) 957-965.

A. Lankoff, J. Bialczyki, D. Dziga, W.W. Carmichael, I. Gradzka, H. Lisowska, T. Kuszewski, S. Gozdz, I. Piorun, A. Wojcik. The repair of gamma-radiation-induced DNA damage is inhibited by microcystin-LR, the PP1 and PP2A phosphatase inhibitor, Mutagenesis 21 (2006b) 83-90.

A. Lankoff, A. Wojcik, V. Fessard, J. Meriluoto. Nodularin-induced genotoxicity following oxidative DNA damage and aneuploidy in HepG2 cells, Toxicology Letters 164 (2006c) 239-248.

H. Li, P. Xie, G. Li, L. Hao, Q. Xiong. *In vivo* study on the effects of microcystin extracts on the expression profiles of proto-oncogenes (*c-fos*, *c-jun* and *c-myc*) in liver, kidney and testis of male Wistar rats injected i.v. with toxins, Toxicon 53 (2009) 169-175.

I.Maatouk, N. Bouaïcha, M.J. Plessis, F. Périn. Detection by 32P-postlabelling of 8-oxo-7,8-dihydro-2'-deoxyguanosine in DNA as biomarker of microcystin-LR- and nodularin-induced DNA damage in vitro *in vitro* in primary cultured rat hepatocytes and *in vivo* in rat liver, Mut. Res. 564 (2004) 9-20.

M. Maidana, V. Carlis, F.G. Galhardi, J.S. Yunes, L.A. Geracitano, J.M. Monserrat, D.M. Barros. Effects of microcystins over short- and long-term memory and oxidative stress generation in hippocampus of rats, Chemico-Biological Interactions 159 (2006) 223-234.

J. Mankiewicz, Z. Walter, M. Tarczynska, O. Palyvoda, M. Staniaszczyk, M. Zalewski. (2002) Genotoxicity of cyanobacterial extracts containing microcystins from Polish water reservoirs as determined by SOS Chromotest and Comet Assay, Environ. Toxicol. 17 (2002) 341-350.

R. Matsushima, S. Yoshizawa, M.F. Watanabe, K. Harada, M. Furusawa, W.W. Carmichael, H. Fujiki. *In vitro* and *in vivo* effects of protein phosphatase inhibitors, microcystins and nodularin, on mouse skin and fibroblasts, Biochem.Biophys. Res. Comm. 171 (1990) 867-874.

A. Milutinović, B. Sedmark, I. Horvat-ŽnidarŠić, D. Šuput. Renal injuries induced by chronic intoxication with microcystins, Cell. Mol. Biol. Lett. 7(2002) 139-141.

A. Milutinović, M. Živin, R. Zorc-Plesković, B. Sedmark, D. Šuput. Nephrotoxic effects of chronic administration of microcystins-LR and -YR, Toxicon 42 (2003) 281-288.

R. Nishiwaki-Matsushima, T. Ohta, S. Nishiwaki, M. Suganuma, K. Kohyama, T. Ishikawa, W.W. Carmichael, H. Fujiki. Liver tumor promotion by the cyanobacterial cyclic peptide toxin microcystin-LR. J. Cancer Res. Clin. Oncol. 118 (1992) 420–424.

A.C.L. Nobre, M.C.M. Jorge, D.B. Menezes, M.C. Fonteles, H.S.A. Monteiro. Effects of microcystin-LR in isolated perfused rat kidney, Brazilian Journal of Medical and Biological Research 32 (1999) 985-988.

Q. Nong, M. Komatsu, K. Izumo, H.P. Indo, B. Xu, K. Aoyama, H.J. Majima, M. Horiuchi, K. Morimoto, T. Takeuchi. Involvment of reactive oxigen species in microcystin-LR-induced cytogenotoxicity. Free Radical Res. 41 (2007) 1326-1337.

E.M. Parry, J.M. Parry, C. Corso, A. Doherty, F. Haddad, T.F. Hermine, G. Johnson, M. Kayani, E. Quick, T. Warr, J. Williamson. Detection and characterization of mechanisms of action of aneugenic chemicals, Mutagenesis 17 (2002) 509-521.

P.V. Rao, R. Bhattacharya. The cyanobacterial toxin microcystin-LR indeced DNA damage in mouse liver *in vivo*, Toxicology 114 (1996) 29-36.

P.V. Rao, R. Bhattacharya, M. Parida, A. Jana, A. Bhaskar. Freshwater cyanobacterium Microcystis aeruginosa (UTEX 2385) induced DNA damage *in vivo* and *in vitro*, Environ. Toxicol. Pharmacol. 5 (1998) 1-6.

W.N. Repavich, J.H. Sonzongni, R.E. Standridge, L.F. Wedphohl, L.F. Meisner. Cyanobacteria (blue-green algae) in Wisconsin waters: acute and chronic toxicity, Water Res. 24 (1990) 225-231.

C. Rosefort, E. Fauth, H. Zankl. Micronuclei induced by aneugens and clastogens in mononucleate and binucleate cells using the cytokinesis block assay, Mutagenesis 19 (2004) 277-284.

N.P. Singh, M.T. McCoy, R.R. Tice, E.L. Schneider. A simple technique for quantitation of low levels of DNA damage in individual cells, Exp Cell Res 175 (1988) 184 –191.

R.M. Soares, V.R. Cagido, R.B. Ferraro, J.R. Meyer-Fernandes, P.R.M. Rocco, W.A. Zin, S.M.F.O. Azevedo. Effects of microcystin-LR on mouse lungs, Toxicon 50 (2007) 330-338.

H. Suzuki, M.F. Watanabe, Y. Wu, T. Sugita, K. Kita, T. Sato, X. Wang, H. Tanzawa, S. Sekiya, N. Suzuki. Mutagenecity of microcystin-LR in human Rsa cells, Int. J. Mol. Med. 2 (1998) 109-112.

R.R. Tice, E. Agurell, D. Anderson, D. Burlinson, A. Hartmann, H. Kobayashi, Y. Miyamae, E. Rojas, J.C. Ryu, Y.F. Sasaki. Single cell gel /comet assay : Guidelines for *in vitro* and *in vivo* genetic toxicology testing, Environ. Mol. Mutagen. 35 (2000) 206-221.

D.M. Toivola, J.E. Eriksson. Toxins affecting cell signaling and alteration of cytoskeletal structure, Toxicol. *In Vitro* 13 (1999) 521-530.

R. Tournebize, S. Andersen, F. Verde, M. Dorée, E. Karsenti, A. Hyman. Distinct roles of PP1 and PP2A-like phosphatases in control of microtubule dynamics during mitosis, The Embo journal 16 (1997) 5537-5549.

K. Tsuji, T. Watanuki, F. Kondo, M.F. Watanabe, S. Suzuki, H. Nakazawa, M. Suzuki, H. Uchida, K. Harada. Stability of microcystins from cyanobacteria-II. Effect of UV on decomposition and isomeration, Toxicon. 33 (1995) 1619-1631.

Y. Ueno, S. Nagata, T. Tsutsumi, A. Hasegawa, M.F. Watanabe, H.D. Park, G.C. Chen, G. Chen, S.Z., Yu. Detection of microcystins, a blue-green algal hepatotoxins, in drinking water sampled in Haimen and Fusui, endemic areas of primary liver cancer in China, by highly sensitive immunoassay, Carcinogenesis 17(1996) 1317-1321.

I. Valentin-Severin, L. Le Hegarat, J.C. Lhuguenot, A.M. Le Bon, M.C. Changon. Use of HepG2 cell line for direct or indirect mutagens screening: comparative investigation between comet and micronucleus assays, Mut. Res. 536 (2003) 79-90.

W.H.O. 1998. Guidelines for drinking water quality, Second Edition, Addendum to Volume 2, Health Criteria and other supporting information, World Health Organization, Geneva.

J.Y. Wu, Q.J. Xu, G. Gao, J.H. Shen. Evaluating genotoxicity associated with microcystin-LR and its risk to source water safety in Meiliang bay, Taihu lake, Environ. Toxicol. 21 (2006) 250-255.

S. Yoshizawa, R. Matsushima, M.F. Watanabe, H. Harada, A. Ichihara, W.W. Carmichael, H. Fujiki. Inhibition of protein phosphatases by microcystis and nodularin associated with hepatotoxicity, J. Cancer Res. Clin. Oncol. 116 (1990) 609-614.

S.Z. Yu. Primary prevention of hepatocellular carcinoma, J. Gastroenterol. Hepatol. 10 (1995) 674-82.

B. Žegura, M. Filipič, D. Šuput, T. Lah, B. Sedmak. In vitro genotoxicity of microcystin-RR on primary cultured hepatocytes and HepG2 cell line detected by Comet assay, Radiol. Oncol. 36 (2002) 159-61.

B. Žegura, B. Sedmak, M. Filipič. Microcystin-LR induces oxidative DNA damage in human hepatoma cell line HepG2, Toxicon 41 (2003) 41-48.

B. Žegura, T. Lah, M. Filipič. The role of reactive oxygen species in microcystin-LR-induced DNA damage, Toxicology 200 (2004) 59-68.

B. Žegura, T. Lah, M. Filipič. Alteration of intracellular GSH levels and its role in microcystin-LR-induced DNA damage in human hepatoma HepG2 cells, Mut. Res. 611 (2006) 25-33.

B. Žegura, M. Volčič, T. Lah, M. Filipič. Different sensitivities of human colon adenocarcinoma (CaCo-2), astrocytoma (IPDDC-A2) and lymphoblastoid (NCNC) cell lines to microcystin-LR induced reactive oxygen species and DNA damage, Toxicon 52 (2008a) 518-525.

B. Žegura, I. Zajc, T. Lah, M. Filipič. Patterns of microcystin-LR induced alteration of the expression of genes involved in response to DNA damage and apoptosis, Toxicon 51 (2008b) 615-623.

L. Zhan, H. Sakamoto, M. Sakuraba, S. Wu, S. Zhang, T. Suzuki, M. Hayashi, M. Honma. Genotoxicity of microcystin-LR in human lymphoblastoid TK6 cells, Mut. Res. 557 (2004) 1-6.

L. Zhou, H. Yu, K. Chen. Relationship between microcystin in drinking water and colorectal cancer, Biomed Environ. Sci.15 (2002) 166-71.

Captions from Figures

Figure 1. Viability of Vero and HepG2 cell lines exposed to MCLR (5 and 20 μM, 24 h) assessed by the NR assay. H2O2 (400 mM/1h) was used as a positive control; mitomycin C (MitC, 0.1 μg/mL, 24h) was tested as the positive control of the micronucleus assay. The results are expressed as the mean percentage of absorbance values relative to the negative control (± standard deviation). Results were obtained from three independent experiments and each treatment condition was tested in triplicate.* represents a statistically significant difference between the treated and the control cells ($p<0.05$).

Figure 2. Results of the micronucleus assay in citokinesis-blocked Vero and HepG2 cells, following exposure to 5 and 20 μM of MCLR for 24 hours. Mitomycin C (MitC, 0.1 μg/mL, 24h) was used as the positive control of the assay. Results are expressed as the mean number of micronucleated binucleated cells (MNBC) (± standard deviation) and were obtained from three (Vero) or two (HepG2) independent experiments, using duplicate cultures.*represents a statistically significant difference between the treated and the control cells ($p<0.05$).

Figure 3. Induced micronucleated binucleated (MNBC) Vero and HepG2 cells calculated by subtracting the frequency of spontaneous MNBC cells from those obtained following MCLR treatment.

Table I. Results of DNA damage (% of tail DNA by the Comet assay) in the Vero and HepG2 cell lines after exposure to MCLR.

Cells	Concentration (μM)		% of DNA in the Comet tail †		
			Exposure time		
			2 h	4 h	24 h
Vero	MCLR	0	3.0 ± 0.3	3.1 ± 0.8	2.5 ± 0.3
		5	3.1 ± 0.3	3.0 ± 0.3	1.9 ± 0.4
		20	2.3 ± 0.4	2.4 ± 0.7	2.4 ± 0.2
	EMS‡	$2x10^4$	43 ± 29	68 ± 15	55 ± 11
HepG2	MCLR	0		2.1 ± 0.3	
		5		2.0 ± 0.1	
		20		2.4 ± 0.3	
	EMS‡	$2x10^4$		53 ± 5	

† Values are the mean (± SD) of three independent experiments.
‡1h of exposure.

Figure 1

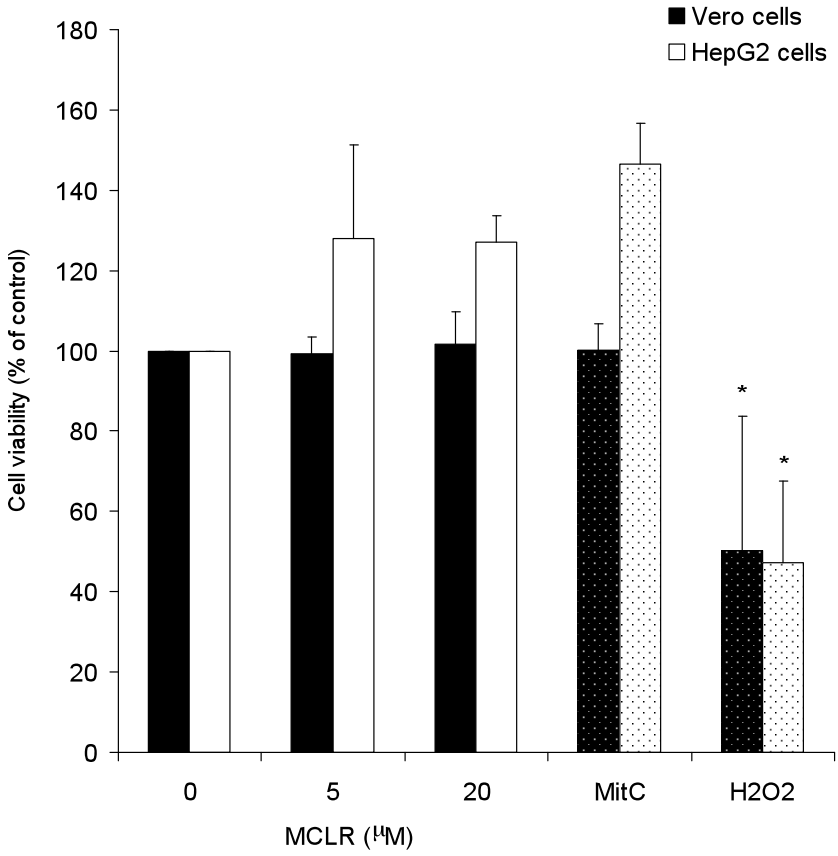

Vero cells
HepG2 cells
Cell viability (% of control)
180
160
140
120
100
80
60
40
20
0
*
*
0
5
20
MitC
H2O2
MCLR (μM)

Figure 2

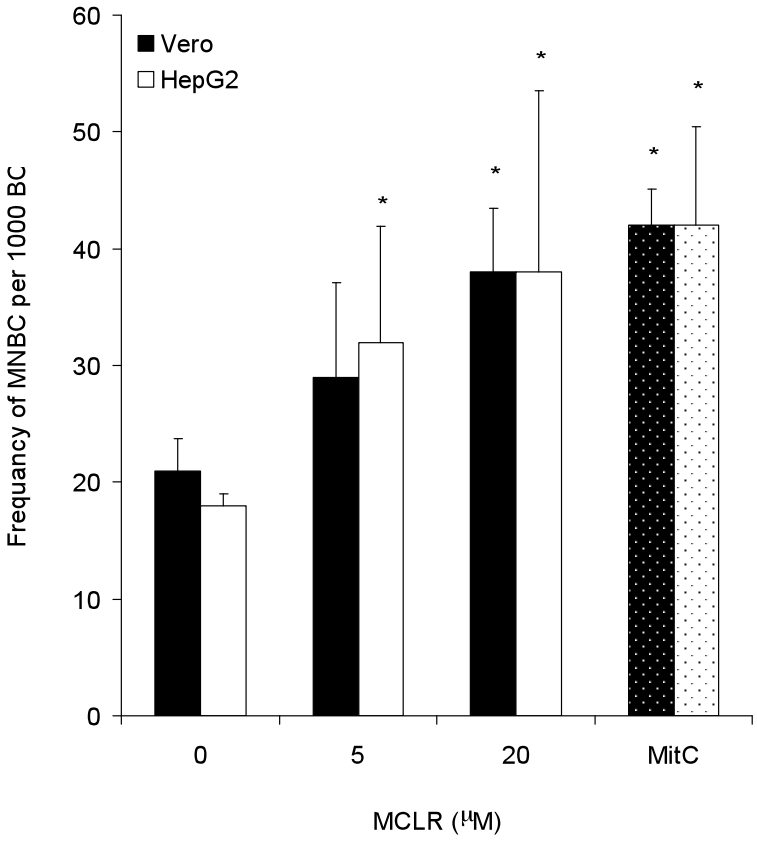
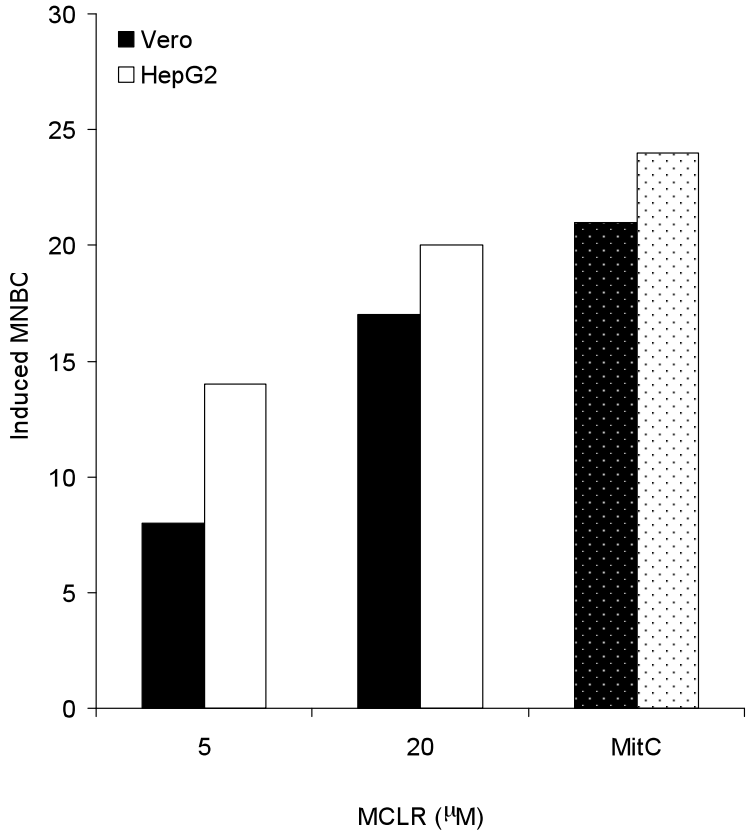

Vero
HepG2
Frequancy of MNBC per 1000 BC
60
50
40
30
20
10
0
0
5
20
MitC
MCLR (μM)
*
*
*
*
*

Figure 3



CAPÍTULO 6

Dias, E., Matos, P., Pereira, P., Batoréu, M.C.C., Silva, M.J., Jordan, P. Microcystin-LR activates the ERK1/2 kinases and stimulates the proliferation of the monkey kidney-derived cell line Vero cells (em preparação para submissão).

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

Authors:

E. Dias[1,2,3,*], P.Matos[1], P.Pereira[2], M.C.C. Batoréu[3], M.J. Silva[1], P. Jordan[1]

Affiliations:

[1] National Institute of Health Dr. Ricardo Jorge, Department of Genetics, Av. Padre Cruz, 1649-016 Lisbon, Portugal

[2], National Institute of Health Dr. Ricardo Jorge, Department of Environmental Health Av. Padre Cruz, 1649-016 Lisbon, Portugal

[3] University of Lisbon, Faculty of Pharmacy, I-Med, Laboratory of Toxicology, Av. Prof. Gama Pinto, 1649-019 Lisbon, Portugal

* Corresponding author - phone: + 351 217519393, fax: + 351 217526400, email: elsa.dias@insa.min-saude.pt

Abstract

Microcystin-LR (MCLR) is a potent hepatotoxin produced by freshwater cyanobacteria. The guideline level for MCLR (1 nM) in drinking water established by the World Health Organization (WHO) was based only on acute toxicological data. Despite increasing evidence suggesting that MCLR may be a potential carcinogen, the available chronic toxicity data on MCLR is yet unsuficient to enable the revision of this value. It is known that MCLR is a potent tumour promoter and it has been proposed that this activity is mediated by the inhibition of protein phosphatases PP1 and 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 characterised the cytotoxicity of MCLR in the kidney derived Vero-E6 cell line and also found genotoxic effects at subcytotoxic concentrations. The purpose of the present work was to evaluate whether the exposure to very low concentrations of MCLR was sufficient to interfere with cell cycle progression and proliferation of Vero cells. Through BrdU incorporation assays we show that even at concentrations below the 1 nM guideline, exposure to MCLR is sufficient to stimulate cell cycle progression in Vero cells. Moreover, the analysis of mitogen activated protein kinases p38, JNK and ERK1/2 activity under these conditions revealed that the proliferative effect of MCLR is associated with the activation of the pro-proliferative ERK1/2 pathway. These results alert to the potential MCLR-induced tumour promotion in the kidney and emphasize the need to review the WHO guideline for MCLR in drinking water.

Keywords: microcystin-LR, tumour promotion, ERK1/2, Vero cells, kidney

Introduction

Microcystins (MC) are secondary metabolites produced by freshwater cyanobacteria that have been associated with severe episodes of human and animal acute hepatotoxicity (Chorus et al., 2000; Duy et al., 2000). 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 the most frequent and toxic variant (Funari and Testai, 2008). MC are produced by several cyanobacteria species from distinct genera such as *Microcystis*, *Anabaena*, *Planktothrix* (Sivonen and Jones, 1999). 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 and haemodialysis treatment are the principal routes for human exposure to MC (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; Fisher 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 acute toxicological data, the World Health Organization (WHO, 1998) has established a guideline for microcystins in drinking water (1 nM of $MCLR_{equiv}$). This guideline constitutes a partial preventive measure to human health since it only accounts for the acute hepatotoxic effects.

In fact, although OATPs are absent, or less expressed, in most non-liver cells (Fisher 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). Moreover, *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). In addition, several studies suggest that MCLR may also have genotoxic activity in several cell types (Ding et al., 1999; Žegura et al., 2004, 2008; Dias et al., 2008), although this is still a matter of some controversy. Nevertheless, MCLR is classified by IARC (2006) as a potential carcinogen to humans (class 2B), though the data collected so far is still insufficient to unequivocally characterize its carcinogenic potential.

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 (Gheringer, 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 Sap1a) 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 e Karin, 2001; Fang and Richardson, 2005; Meloche e 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 have demonstrated that MCLR induces cytotoxic (organelle changes/damages, cell lysis, apoptosis, necrosis) and genotoxic (induction of micronuclei) effects on the kidney-derived Vero-E6 cell line (Dias et al., 2008, 2009 Alverca et al., 2009). These effects were observed within the concentration range of 5 to 20 μM of MCLR, according to a dose and time-dependent manner. The purpose of the present work was to evaluate the effects of low levels of MCLR (within the nM range) on the same cell line regarding the activation status of MAP kinases and the stimulation of cell cycle progression and proliferation.

Materials and Methods

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 -20ºC 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 (LMECYA110 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 eukariotes, non-axenic cultures. The microcystin production by those strains was previously characterized by HPLC-DAD, ELISA and PCR (Valério et al., 2009b). LMECYA110 produces exclusively the MCLR variant while LMECYA127 is a non-microcystin producer. 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 ± 4 $\mu E.m^{-2}.s^{-1}$, aprox.) at 20 ± 1ºC. 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 4ºC 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 35ºC (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, Geramny). The elution fractions (5 mL) were collected on a fraction collector (Bio-Rad Mod. 2110, CA, USA) and analyzed 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 -20ºC 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.

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 cm2 flasks (Nunc, Roskilde, Denmark) in Modified Eagle Medium (MEM) supplemented with 10% FBS, 0.1mM non-essential aminoacids and 1mM sodium pyruvate, in a 5% CO_2 humidified incubator at 37ºC. Cells in exponential growth phase were detached from the growth surface (trypsin, 0.5%), centrifuged (300 x *g*) and the cell viability was determined by the trypan blue dye exclusion method (Philips, 1973).

3. Evaluation of MCLR effect on ERK1/2 activation

3. 1. Cell exposure to cyanobacterial extracts and pure MCLR

Vero cells were seeded in 35 mm culture dishes and cultured for 24 hours 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 0.005 to 5 μM of MCLR. The same biomass dilutions (from 0.56 to 556 $\mu g.mL^{-1}$) of the non-toxic LMECYA127 extract were tested in parallel as a control of *M. aeruginosa* extract matrix. Serial dilutions of pure MCLR stock solution containing 0.005 to 5 μM of toxin were also tested. The cells were exposed to LMECYA extracts and pure MCLR for 24 h prior to MAPK analysis by immunoblloting (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.

3.2 Cell lysis, SDS-PAGE and Western blot analysis of ERK1/2

After toxin exposure, cells were washed with PBS and lysed with 2x Laemmli buffer. Lysates were denaturated at 95ºC for 10 min. and proteins were separated on 10% SDS-polyacrylamide gels. After electrophoresis, proteins were transferred onto

PVDF (polyvinylidene difluoride) membranes (BioRad) 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, 5min) 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, BioRad) by Enhanced ChemiLuminescence (ECL) detection.

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-20-deoxyuridine during DNA synthesis. Vero cells ($2x10^5$) were seeded on 10x10 mm cover slips placed in 35mm 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 4ºC, permeabilized with 100% methanol for 10 min at rt and then incubated with 4N HCl for 10 min to denature DNA. After neutralization with 1M Tris, pH 8.8 cells were washed 3x with PBS, coverslips were incubated with the mouse-anti-BrdU primary antibody (Roche) (dilution 1/20), followed by goat-anti-mouse secondary antibody conjugated with TexasRed (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.

6. 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 $p < 0.05$.

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 cells was analysed by Western-blot using phospho-specific antibodies (figure 1). Interestingly, while all the LMECYA 110 extract dilutions (containing 0.005, 0.05, 0.5 and 5 μM of MCLR) induced a marked increase of phosphorylated ERK1/2 (p-ERK1/2), no considerable variation was found in the phosphorylation state of p38 and JNK (figure 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 (figure 2) and similar concentrations of commercially available MCLR (figure 3). As shown in figure 2, none of the dilutions of the LMECYA 127 extract induced any effect on the activation state of ERK1/2. Exposure of Vero cells to commercial MRLC reproduced an increase in ERK1/2 phosphorylation consistent with the observed with the LMECYA 110 extract (figure 3).

Densitometric analysis of Western-blots from independent experiments with *M. aeruginosa* extracts (figure 4) revealed that exposure to LMECYA 110 extract containing low concentarions of MCLR was sufficient to induce a 1.71 to 3.66 fold increase in p-ERK1/2 with maximum effect at 50 nM. A significant difference 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. The overall effect in p-ERK1/2 was slightly higher for pure MCLR (2.8 to 4 fold increase) and the maximum effect was achieved at a lower toxin concentration (5 nM) as shown by densitometric analysis of Western-blots from 3 independent experiments (figure 5).

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 cells by the G1/S transition BrdU assay. As shown in figure 6, exposure of starved cells (1% FBS) to a range of 1 to 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 (figure 7).

Discussion

In this work we demonstrated that MCLR stimulates the proliferation of kidney-derived Vero-E6 cell line in the concentration range of 1 to 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 nM and 50 nM dose-range, respectively. This indicates that the stimulatory activity of low MCLR concentrations on Vero 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 (Gheringer, 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 cells that low MCLR concentration affected the ERK1/2 but not the p38 and JNK MAPK pathways. 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 immortalised cell lines is reduced or even abolished (Bouaru 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 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 cells within a broad range of concentrations (5 nM – 5 μM), however, cell proliferation was mainly stimulated at a MCLR concentration of only 5 nM. In contrast, we previously demonstrated that MCLR induces autophagy in Vero cells exposed to 5 μM, followed by apoptosis 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-Denis 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 cells: the stimulation of cell proliferation at low MCLR concentrations and the induction of autophagic (pre-apoptotic) mechanisms at (sub)cytotoxic concentrations of MCLR. We also speculate that under the autophagic response, the stimulation of the ERK1/2 pathway may contribute to the inhibition of apoptotic cell death that occurred only at higher toxin concentrations.

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. 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 for toxin concentrations close to the mandatory level of MCLR for drinking water (1 nM). Our results also suggest that the effect of MCLR on cell proliferation is mediated by the activation of the ERK1/2 pathway, which is known to be deregulated in approximately

one-third of all human cancers (Dhillon et al., 2007). These results emphasise the importance to confirm *in vivo* the impact of MCLR on tumour promotion at kidney level.

Acknowledgements

This work was supported by the Portuguese Foundation for Science and Technology via grant SFRH/BD/10585/2002 attributed to Elsa Dias and plurianual programs for the Centre for Research on Human Molecular Genetics (CIGMH) and Institute for Medicine and Pharmaceutical Sciences (I-MED).

References

Alverca, E., et al., Morphological and ultrastructural effects of microcystin-LR from *Microcystis aeruginosa* extract on a kidney cell line, Toxicon (2009), doi: 10.1016/j.toxicon.2009.04.014.

Boaru, D.A., Dragoş, N., Schirmer, K. 2006. Microcystin-LR induced cellular effects in mammalian and fish primary hepatocyte cultures and cell lines: A comparative study. Toxicology 218: 134-148.

Botha, N., Van de Venter, M., Downing, T.G., Shepard, E.G., Gehringer, E.G. 2004. The effect of intraperitoneally administered microcystin-LR on the gastrointestinal tract of Balb/c mice. Toxicon 43: 251-254.

Chang, L., Karin, M. 2001. Mammalian MAP kinase signalling cascades. Nature 410: 37-40.

Chen, Y.M., Lee, T.H., Lee, S.J., Huang, H.B., Huang, R., Chou, H.N. 2006. Comparison of protein phosphatase inhibition activities and mouse toxicities of microcystins. Toxicon 47: 742-746.

Chorus, I., Falconer, I.R., Salas, H.J., Bartram, J. 2000. Health risks caused by freshwater cyanobacteria in recreational waters. J. Toxicol. Environ. Health, Part B 3: 323-347.

Codd, G.A. 2000. Cyanobacterial toxins, the perception of water quality, and the priorisation of eutrophication control. Ecol. Eng. 16: 51-60.

Codogno, P., Meijer, A.J. 2005. Autophagy and signaling: their role in cell survival and death. Cell Death Different. 12: 1509-1518.

Dhillon, A.S., Hagan, S., Rath, O., Kolch, W. 2007. MAP kinase signalling pathways in cancer. Oncogene 26: 3279-3290.

Dias, E., Pereira, P., Batoréu, M.C.C., Jordan, P., Silva, M.J. 2008. Cytotoxic and genotoxic effects of microcystins in mammalian cell lines. In: Ø. Moestrup, et al. (Eds.), Proceedings of the 12th International Conference on Harmful Algae, ISSHA and IOC- UNESCO, Paris, pp. 282-285.

Dias, E., Andrade, M., Alverca, E., Pereira, P., Batoréu, M.C.C., Jordan, P., Silva, M.J. 2009. Comparative study of the cytotoxic effect of microcistin-LR and purified extracts from *Microcystis aeruginosa* on a kidney cell line. Toxicon 53: 487-495.

Ding, W.X., Shen, H.M., Zhu, H.G., Lee, B.L., Ong, C.N. 1999. Genotoxicity of microcistic cyanobacterial extract of a water source in China. Mutat Res 442: 69-77.

Ding, X.S., Li, X.Y., Duan, H.Y., Chung, I.K., Lee, J.A. 2006. Toxic effects of Microcystis cell extracts on the reproductive system of male mice. Toxicon 48: 973-979.

Duy, T.N., Lam, P.K.S., Shaw, G.R., Connell, D.W. 2000. Toxicology and risk assessment of freshwater cyanobacterial (blue-green algae) toxins in water. Rev. Environ. Contam. Toxicol. 163: 113-186.

Eskelinen, E.L., Saftig, P. 2009. Autophagy: A lysosomal degradation pathway with a central role in health and disease. Biochem. Biophys. Acta 1793: 664-673.

Falconer, I.R. 1991. Tumour promotion and liver injury caused by oral consumption of cyanobacteria, Environ. Toxicol. Water Qual. 6: 177-184.

Falconer, I.R., Yeung, D.S. 1992. Cytoskeletal changes in hepatocytes induced by microcystis toxins and their relation to hyperphosphorilation of cell proteins, Chem. Biol. Interact. 81: 181-196.

Fang, J.L., Richardson, B.C. 2005. The MAPK signalling pathways and colorectal cancer. Lancet Oncol. 6: 322-327.

Fischer, W.L, Altheimer, S., Cattori, V., Meier, P.J., Dietrich, D.R., Hagenbuch, B. 2005. Organic anion transporting polypeptides expressed in liver and brain mediate uptake of microcystin. Toxicol. Appl. Pharmacol. 203: 257-263.

Funari, E., Testai, E. 2008. Human health risk assessment related to cyanotoxin exposure. Critical Rev. Toxicol. 38: 97-125.

Gaudin, J., Huet, S., Jarry, G., Fessard, V. 2008. *In vivo* DNA damage induced by the cyanotoxin microcystin-LR: comparison of intra-peritonial and oral administration by use of the comet assay. Mutat. Res. 652: 65-71.

Gehringer, M.M. 2004. Microcystin-LR and okadaic acid-induced cellular effects: a dualistic response. FEBS Letters 557, 1-8.

Gilley, R., March, H.N., Cook, S.J. 2009. ERK1/2, but not ERK5, is necessary and sufficient for phosphorylation and activation of c-Fos.Cellular Signal. 21: 969-977.

Hagenbuch, B., Meier, P.J. 2003. The superfamily of organic anion transporting polypeptides, Biochimica et Biophysica Acta 1609: 1-18.

Humpage, A.R., Hardy, S.J., Moore, E.J., Froscio, S.M., Falconer, I.R. 2000. Microcystins (cyanobacterial toxins) in drinking water enhance the growth of aberrant crypt foci in the mouse colon. J. Toxicol Environm Health A 61: 155-65.

IARC. 2006. Cyanobacterial peptide toxins. Available at: http://monographs.iarc.fr/ ENG/Meetings/94-cyanobacteria.pdf.

ISO 20179, Water quality – determination of microcystins – method using solid phase extraction (SPE) and high performance liquid chromatography (HPLC) with ultraviolet (UV) detection.

Ito, E., Takai, A., Kondo, F., Masui, H., Imanishi, S., Harada, K.I. 2002. Comparison of protein phosphatase inhibitory activity and apparent toxicity of microcystins and related compounds. Toxicon 40: 1017-1025.

Junttila, M.R., Li, S.P., Westermarck, J. 2008. Phosphatase-mediated crosstalk between MAPK signalling pathways in the regulation of cell survival. FASEB J. 22: 954-965.

Komatsu, M., Furukawa, T., Ikeda, R., Takumi, S., Nong, Q., Aoyama, K., Akiyama, S.I., Keppler, D., Takeuchi, T. 2007. Involvment of mitogen-activated protein kinase signalling pathways in microcystins-LR-induced apoptosis after its selective uptake mediated by OATP1B1 and OATP1B3. ToxicolSci. 97: 407-416.

Li, H., Xie, P., Li, G., Hao, L., Xiong, Q. 2009. *In vivo* study on the effects of microcystin extracts on the expression profiles of proto-oncogenes (*c-fos*, *c-jun* and *c-myc*) in liver, kidney and testis of male Wistar rats injected i.v. with toxins. Toxicon 53: 169-175.

Maidana, M., Carlis, V., Galhardi, F.G., Yunes, J.S., Geracitano, L.A., Monserrat, J.M., Barros, D.M. 2006. Effects of microcystins over short- and long-term memory and oxidative stress generation in hippocampus of rats. Chemico-Biological Interactions 159: 223-234.

Meijer, A.J., Codogno, P. 2004. Regulation and role of autophagy in mammalian cells. Int. J. Biochem. Cell Biol. 36: 2445-2462.

Meloche, S., Pouysségur, J. 2007. The ERK1/2 mitogen-activated protein kinase pathway as a master regulator of the G1- to S-phase transition. Oncogene 26: 3227-3239.

Milutinović, A., Sedmark, B., Horvat-ŽnidarŠić, I., Šuput, D. 2002. Renal injuries induced by chronic intoxication with microcystins. Cell. Mol. Biol. Lett. 7: 139-141.

Milutinović, A., Živin, M., Zorc-Plesković, R., Sedmark, B., Šuput, D. 2003. Nephrotoxic effects of chronic administration of microcystins-LR and –YR. Toxicon 42: 281-288.

Nishiwaki-Matsushima, R., Ohta, T., Nishiwaki, S., Suganuma, M., Kohyama, K., Ishikawa, T., Carmichael, W.W., Fujiki, H. 1992. Liver tumor promotion by the cyanobacterial cyclic peptide toxin microcystin-LR. J. Cancer Res. Clin. Oncol. 118: 420–424.

Nobre, A.C.L., Jorge, M.C.M., Menezes, D.B., Fonteles, M.C., Monteiro, H.S.A. 1999. Effects of microcystin-LR in isolated perfused rat kidney. Brazilian Journal of Medical and Biological Research 32: 985-988.

Ogier-Nenis, E., Pattingre, S., Benna, J.E., Codogno, P. 2000. Erk1/2-dependent phosphorilation of Gα-interacting protein stimulates its GTPase accelerating activity and autophagy in human colon cancer cells. J. Biol. Chem. 275: 39090-39095.

Pattingre, S., Bauvy, C., Codogno, P. 2003. Aminoacids interfere with the Erk1/2-dependent control of macroautophagy by controlling the activation of Raf-1 in human colon cancer HT-29 cells. J. Biol. Chem. 278: 16667-16674.

Philips, H.J. 1973. Dye exclusion tests for cell viability. *In*: Kruse, P.F. & Patterson, M.K. (Eds.) Tissue Culture: Methods and Applications. Academic Press, NY, pp. 406-408.

Robinson N.A., Pace J.G., Matson C.F., Miura G.A., Lawrence W.B. 1990. Tissue distribution, excretion and hepatic biotransformation of microcystin-LR in mice. J. Pharmacol.Exp. Ther. 256:176-182.

Sivonen, K., Jones, G. 1999. Cyanobacterial toxins. In: I. Chorus, J.Bartram (Eds.) Toxic cyanobacteria in water: a guide to their public health consequences, monitoring and management, E & FN Spon, London, pp.41-111.

Soares, M., Cagido, V.R., Ferraro, R.B., Meyer-Fernandes, J.R., Rocco, P.R.M., Zin, W.A., Azevedo, S.M.F.O. 2007. Effects of microcystin-LR on mouse lungs. Toxicon 50: 330-338.

Turatti, E., Neves, A.C, Magalhães, M.H.C.G., Sousa, S.O.M. 2005. Assessment of c-Jun, c-Fos and cyclin D1 in premalignant and malignant oral lesions. J. Oral Sci. 47: 71-76.

Ueno, Y., Nagata, S., Tsutsumi, T., Hasegawa ,A., Watanabe, M.F., Park, H.D., Chen, G.C., Chen, G., Yu, S.Z. 1996. Detection of microcystins, a blue-green algal hepatotoxins, in drinking water sampled in Haimen and Fusui, endemic areas of primary liver cancer in China, by highly sensitive immunoassay. *Carcinogenesis* 17: 1317-1321.

Valério, E., Chambel, L., Faria, N., Paulino, S., Pereira, P., Tenreiro, R. 2009a. Molecular identification, typing and traceability of cyanobacteria from freshwater reservoirs. Microbiology 155: 642 – 656.

Valério, E. et al, Multiplex PCR for detection of microcystins-producing cyanobacteria from freshwater samples, Environ. Toxicol. (2009b), doi: 10.1002/Tox.20502.

van Apeldoorn, M.E., van Egmond, H.P., Speijers, G.J.A., Bakker, G.J.I. 2007. Toxins of cyanobacteria. Mol. Nutr. Food Res. 51: 7-60.

W.H.O. 1998. Guidelines for drinking water quality, Second Edition, Addendum to Volume 2, Health Criteria and other supporting information. World Health Organization, Geneva.

Yoshizawa, I., Matsushima, R., Watanabe, M.F., Harada, H., Ichihara, A., Carmichael, W.W., Fujiki, H. 1990. Inhibition of protein phosphatases by microcystis and nodularin associated with hepatotoxicity. J. Cancer Res. Clin. Oncol. 116: 609-614.

Yu, S.Z. 1995. Primary prevention of hepatocellular carcinoma, J. Gastroenterol. Hepatol. 10: 674-82.

Žegura, B., Lah, T., Filipič, M. 2004. The role of reactive oxygen species in microcystin-LR-induced DNA damage. Toxicology 200 : 59-68.

Žegura, B., Volčič, M., Lah, T., Filipič, M. 2008. Different sensitivities of human colon adenocarcinoma (CaCo-2), astrocytoma (IPDDC-A2) and lymphoblastoid (NCNC) cell lines to microcystin-LR induced reactive oxygen species and DNA damage. Toxicon 52: 518-525.

Zhou, L., Yu, H., Chen K. 2002. Relationship between microcystin in drinking water and colorectal cancer. Biomed Environ. Sci.15: 166-71.

Zhu, Y., Zhong, X., Zheng, S., Ge, Z., Du, Q., Zhang, S. 2005. Transformation of immortalized colorectal crypt cells by microcystin involving constitutive activation of Akt and MAPK cascade. Carcinogenesis 26: 1207-1214.

Captions from figures

Figure 1. Activation status of ERK1/2, p38 and JNK kinases in Vero cells by a MCLR-producer *M. aeruginosa* strain (LMECYA 110). Cells were exposed for 24 h to LMECYA 110 extract dilutions containing 0.005, 0.05, 0.5 and 5 μM 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 cells treated for Epidermal Growth Factor (EGF, 10 $ng.mL^{-1}$, 5 min) for ERK1/2 analysis and of cells treated with anisomycin (0.25 μM, 1h) for JNK and p38 analysis. Results were obtained from two independent experiments.

Figure 2. Activation status of ERK1/2 kinases in Vero cells by a non-MCLR producer *M. aeruginosa* strain (LMECYA 127). Cells were exposed for 24 h to LMECYA 127 extract dilutions containing biomass concentration equivalent to LMECYA 110 (0.56, 5.6, 56 and 560 μg $dw.mL^{-1}$). The levels of active (phosphorylated) and total ERK1/2 kinases were detected by Western-Blot. The negative control consisted in non-treated cells. The positive control consisted of cells treated with EGF (10 $ng.mL^{-1}$, 5 min). Results were obtained from two independent experiments.

Figure 3. Activation of the ERK1/2 kinases by low doses of pure MCLR in Vero cells. Cells were exposed for 24 h to MCLR (0.005, 0.005, 0.05, 0.5 and 5 μM) prior to Western-Blot analysis of active (phosphorylated) and total ERK1/2 levels (negative and positive controls as described for figure 2). Results were obtained from three independent experiments.

Figure 4. Activation of ERK1/2 in Vero cells exposed to *M. aeruginosa* extracts LMECYA 110 (MCLR-producer strain) and LMECYA 127 (non-toxic strain), calculated by densitometry analysis of Western blots. The results are presented as mean ± standard deviation of treated cells in relation to negative control. Secondary x-axis represents the *M. aeruginosa* biomass concentration. *p* indicates the significance of the difference between the two dose-response curves by a paired Student's *t*-test.

Figure 5. Activation of ERK1/2 in Vero cells exposed to pure MCLR, calculated by densitometry analysis of the bands obtained from Western blot analyses. The results are presented as mean ± standard deviation of treated cells in relation to negative control. * represents a statistically significant difference between treated and control cells ($p<0.05$).

Figure 6. Effect of pure MCLR on cell cycle progression of Vero 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.

Figure 7. Quantification of the effect of pure MCLR (0.5, 1, 5 and 10 nM) on Vero 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.

Figure 1

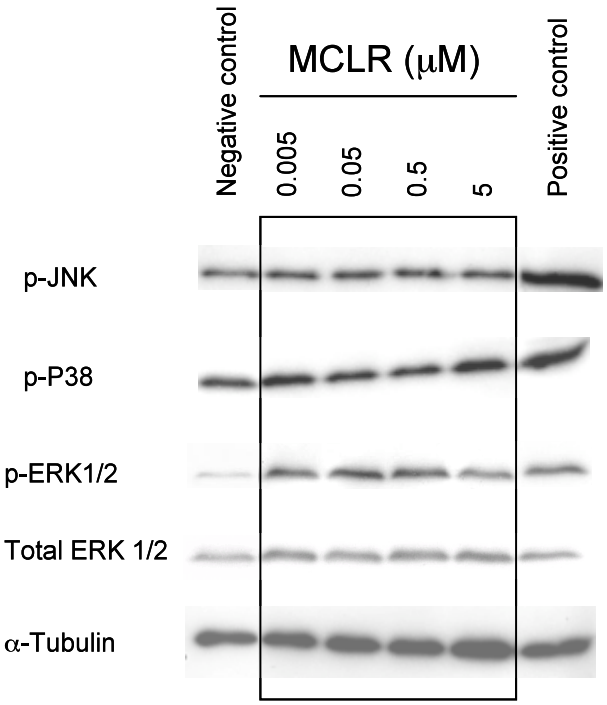


Figure 2

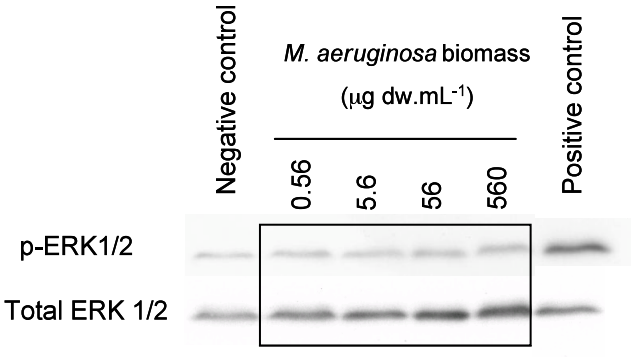


Figure 3

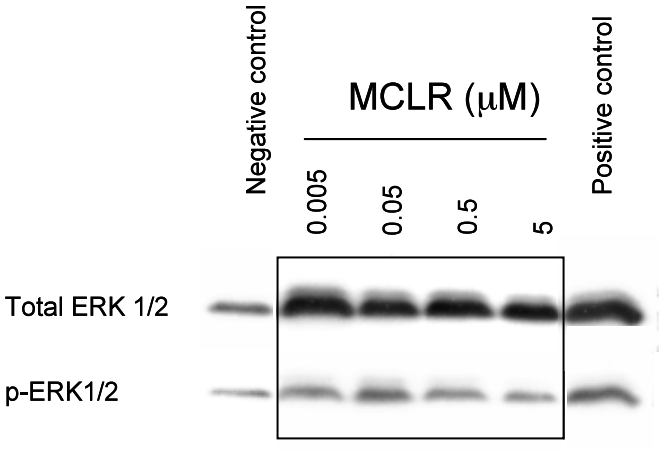


Figure 4

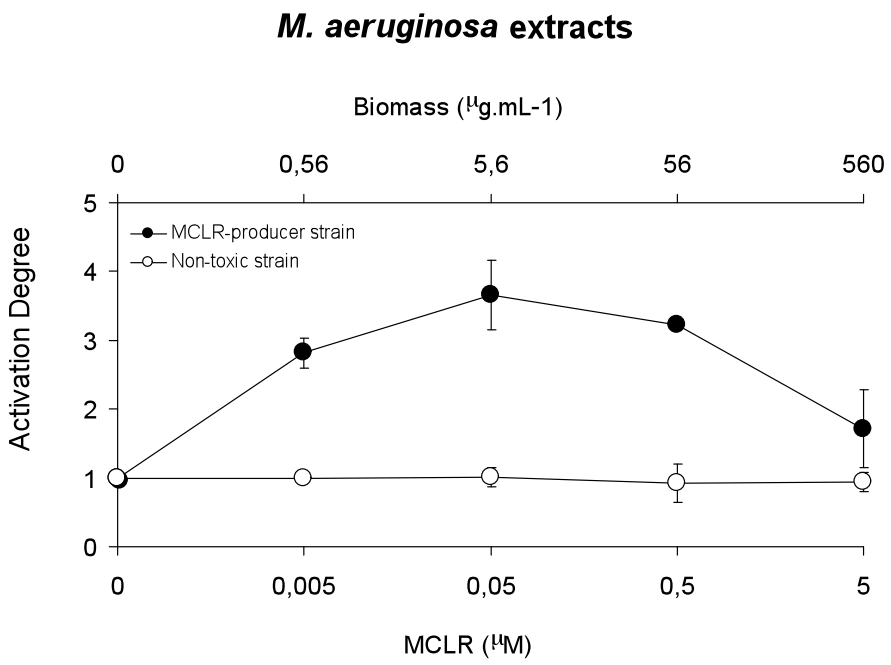


Figure 5

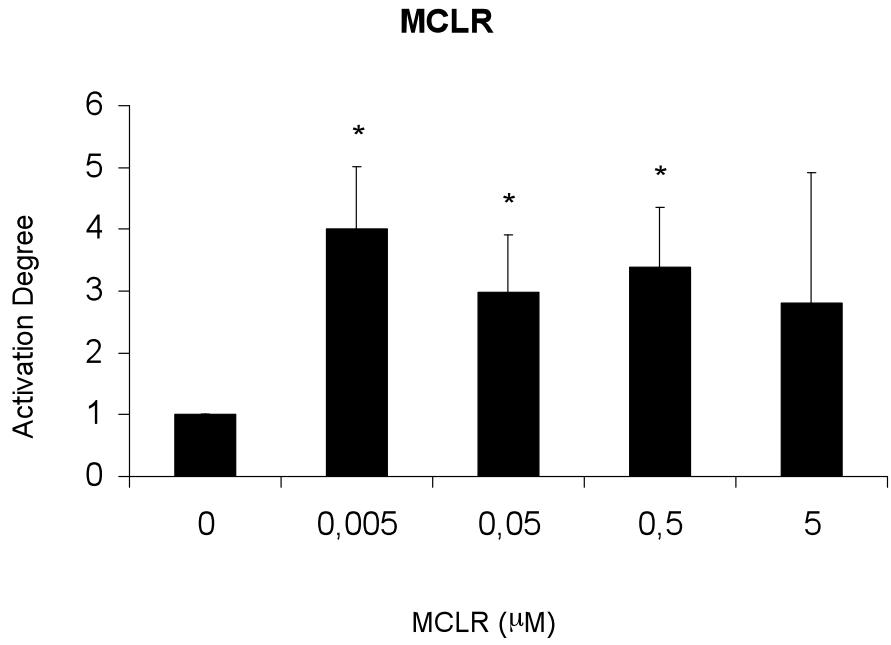


Figure 6

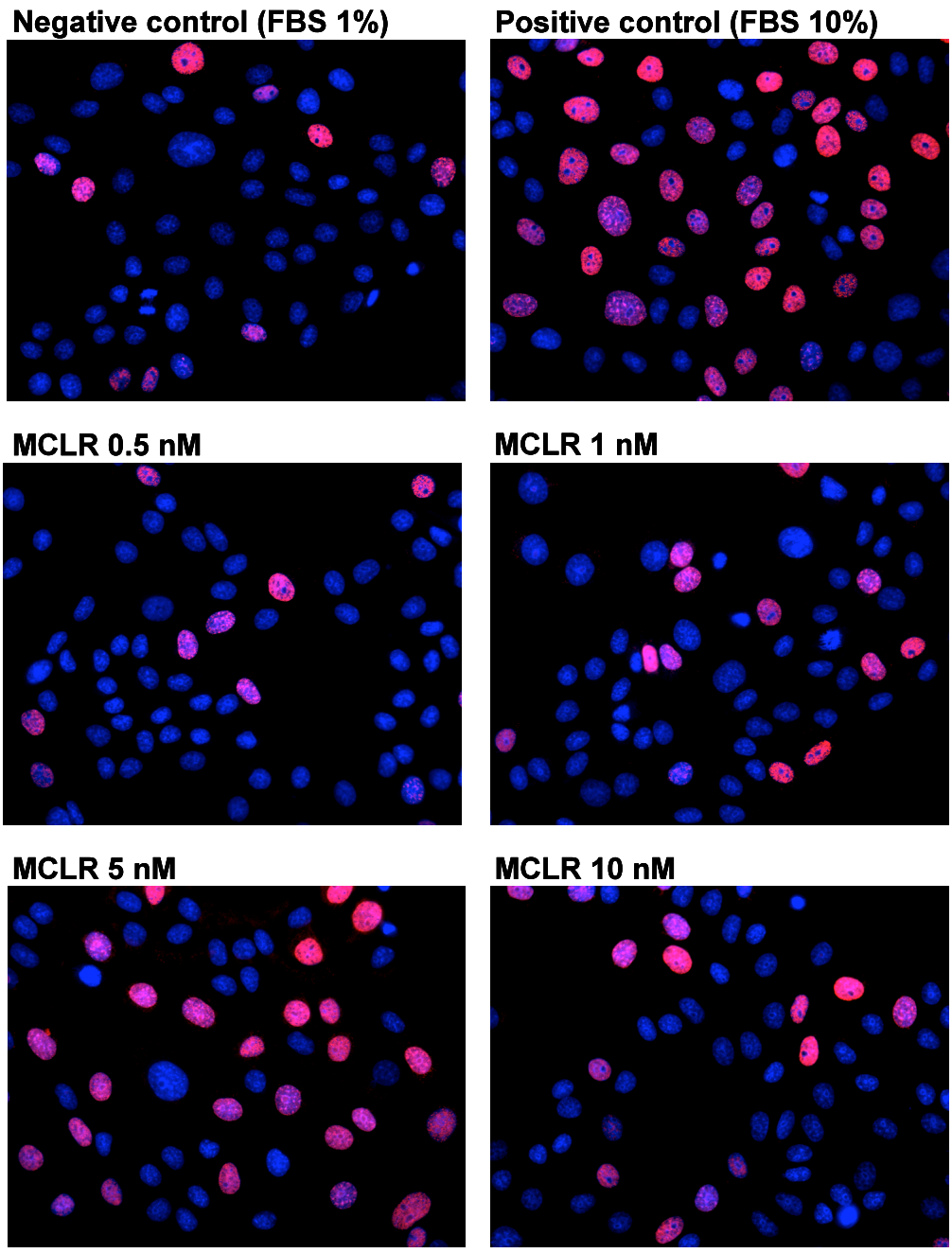
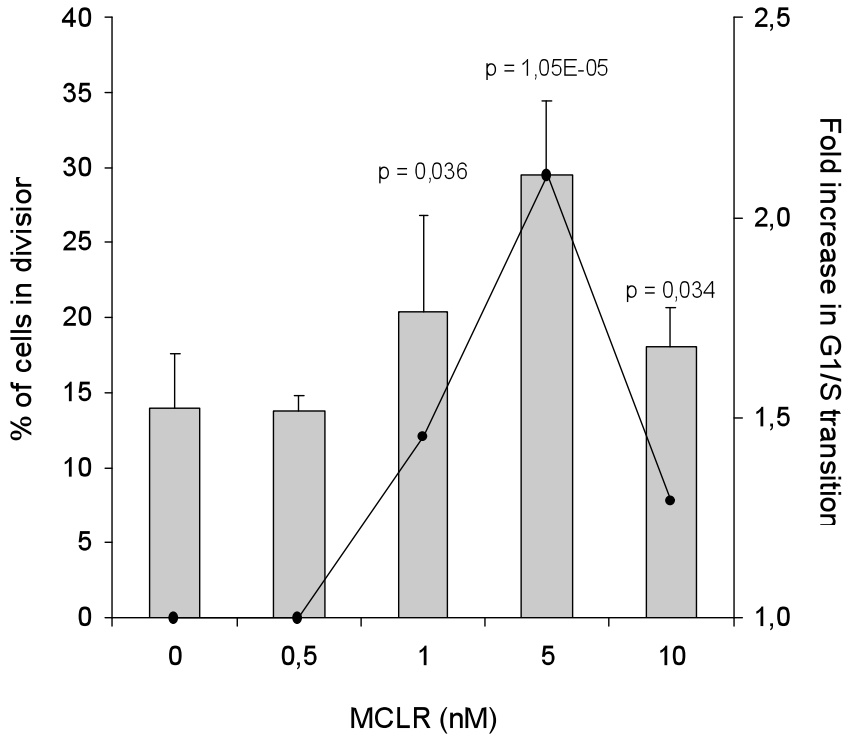

Negative control (FBS 1%)
Positive control (FBS 10%)
MCLR 0.5 nM
MCLR 1 nM
MCLR 5 nM
MCLR 10 nM

Figure 7


40
35
30
25
20
15
10
5
0
% of cells in divisior
2,5
2,0
1,5
1,0
Fold increase in G1/S transition
p = 1,05E-05
p = 0,036
p = 0,034
0
0,5
1
5
10
MCLR (nM)

CAPÍTULO 7

DISCUSSÃO GERAL E CONCLUSÕES

Este trabalho teve como principal objectivo a avaliação do potencial cancerigénico da MCLR no modelo experimental Vero-E6 (linha celular epitelial derivada do rim de Macaco Verde Africano). Os estudos toxicológicos *in vitro* com a MCLR têm sido conduzidos maioritariamente em hepatócitos, uma vez que o fígado é o órgão-alvo principal da toxicidade aguda das microcistinas. Contudo, estudos recentes mostram que outros órgãos, tais como o rim (Nobre et al., 1999; Milutinović et al., 2002, 2003; Moreno et al., 2005; Andrinolo et al., 2008), também estão sujeitos à acção tóxica da MCLR. Embora a eliminação desta toxina seja efectuada sobretudo pela via biliar, uma pequena percentagem (9%) é também excretada por via urinária (Robinson et al., 1990), pelo que os potenciais efeitos nocivos da MCLR ao nível renal não deverão ser negligenciados. A discussão sobre o impacto das microcistinas noutros órgãos é importante em termos de saúde pública, uma vez que as medidas preventivas associadas à ocorrência de microcistinas em água de consumo humano baseiam-se no valor-guia da OMS (1 nM) que, por sua vez, foi calculado com base apenas na hepatotoxicidade aguda da MCLR (WHO, 1998). Assim, aquelas medidas poderão não contemplar a toxicidade total das microcistinas no organismo uma vez que excluem a sua toxicidade noutros órgãos para além do fígado. Por outro lado, não consideram os efeitos decorrentes da exposição continuada a baixas doses de microcistinas, em particular os seus potenciais efeitos cancerigénicos.

1. A multiplicidade de efeitos da MCLR na linha celular Vero-E6.

Os resultados obtidos neste trabalho demonstram que a MCLR desencadeia uma multiplicidade de efeitos na linha celular Vero-E6. Nos capítulos 2 a 6 da presente dissertação descreveram-se efeitos a vários níveis usando abordagens metodológicas diversas, tal como se indica na tabela 1.

Tabela 1. Efeitos induzidos pela MCLR na linha celular Vero-E6.

Efeito	Concentração de MCLR (24h de exposição)	Abordagem metodológica	Capítulo
Citotoxicidade	25 – 200 μM	Testes de viabilidade celular (NR, MTT e LDH)	2 e 3
Autofagia Apoptose Necrose	5 – 10 μM 20 – 40 μM 75 μM	Microscopia Electrónica de Transmissão/ Imunofluorescência / Teste de *Tunel*	4
Genotoxicidade	5 – 20 μM	Ensaio do Micronúcleo	2 e 5
Proliferação celular	1– 10 nM	Teste de incorporação de BrdU Analise da expressão de MAPK	6

No seu conjunto, os resultados obtidos neste trabalho permitiram estabelecer uma relação entre a dose de MCLR e o tipo de efeito nas células Vero-E6. De facto, para doses na gama de 1-10 nM a MCLR estimula a proliferação celular através da activação da via de sinalização ERK1/2. Para concentrações na ordem dos μM a MCLR desencadeia uma variedade de efeitos em praticamente todos os compartimentos celulares, desde efeitos genotóxicos (indução de micronúcleos) e autofagia até a efeitos conducentes à morte celular por apoptose ou necrose (figura 1).

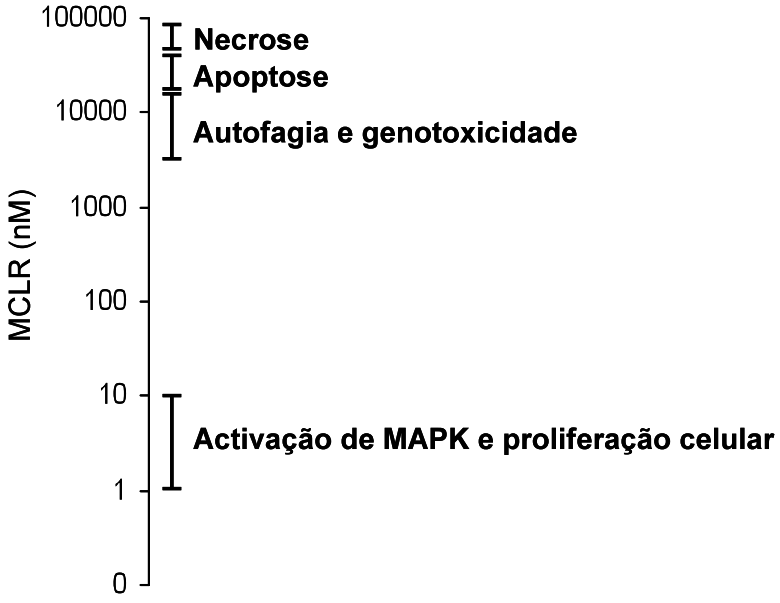


Figura 1. Relação dose-efeito da MCLR na linha celular Vero-E6.

Embora alguns destes efeitos possam ocorrer simultaneamente em função do binómio dose/tempo de exposição, poder-se-à inferir, no entanto, uma sequência de eventos tóxicos induzidos pela MCLR, sobretudo no que diz respeito aos efeitos citotóxicos (perda de viabilidade celular). Primeiro, e talvez numa estratégia de sobrevivência, as células desencadeiam mecanismos de autofagia, tal como pode ser deduzido pelo aumento do número de autofagossomas e pelas alterações ao nível do sistema Golgi-Retículo Endoplasmático (capítulo 4). Mantendo-se o estímulo tóxico a níveis elevados, e não sendo suficiente a estratégia anterior para eliminar o efeito da MCLR, parecem ser então desencadeados mecanismos conducentes à morte celular programada (apoptose). Este efeito foi determinado pelo teste de Tunel e corroborado pelas alterações morfológicas e ultrastruturais ao nível da mitocôndria, citosqueleto e do núcleo (capítulo 4). Para condições de exposição extremas os mecanismos de sobrevivência não serão eficazes e a toxicidade da MCLR manifesta-se "descontroladamente", levando à total desorganização da célula e morte celular por necrose, tal como foi observado ao nível da ultrastrutura e corroborado pelos testes de viabilidade celular (capítulos 3 e 4).

A MCLR desencadeia, portanto, um efeito dual na linha celular Vero-E6: a doses baixas constitui um estímulo positivo relativamente ao crescimento celular, enquanto que a doses altas induz processos conducentes à inviabilidade e morte celular (figura 2).

O facto da MCLR induzir uma diversidade de efeitos na linha celular Vero-E6 e do seu impacto no crescimento celular poder ser descrito por uma função dose/resposta, sugere que esta linha celular é um modelo celular adequado para o estudo da nefrotoxicidade das microcistinas

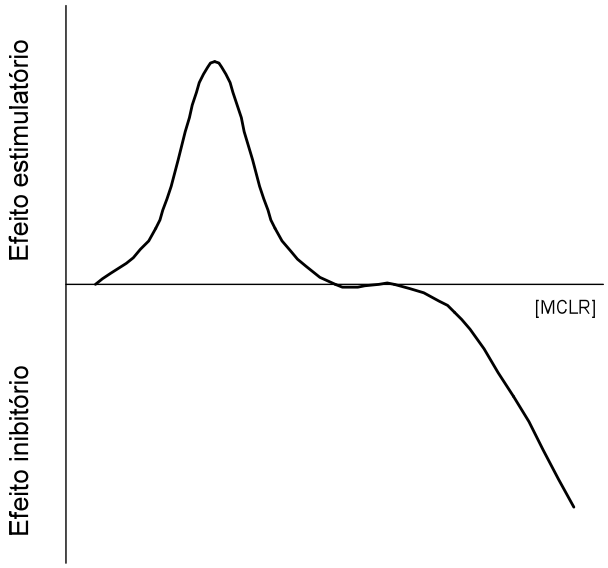


Figura 2. Efeito dual da MCLR no crescimento da linha celular Vero-E6 em função da concentração.

2. O papel da MCLR no processo de cancerigénese.

O papel das microcistinas no processo de cancerigénese está ainda por esclarecer. Alguns estudos em murganhos *in vivo* permitem concluir que a MCLR promove a formação/crescimento tumoral no fígado (Nishiwaki-Matsushima et al. 1992), na pele (Falconer, 1991) e no cólon (Ito et al., 1997; Humpage et al., 2000a). Contudo, a informação acerca dos mecanismos responsáveis por essa actividade resume-se a um número de publicações muito diminuto (Gheringer, 2004; Zhu et al., 2005; Komatsu et al., 2007), que sugerem o envolvimento de cascatas de sinalização da família das MAPK. Os resultados descritos no capítulo 6 reforçam esta hipótese, uma vez que mostram que a MCLR estimula a proliferação celular no modelo Vero-E6 através da activação da via de sinalização ERK1/2. Estes resultados são particularmente interessantes, uma vez que os efeitos observados ocorreram a doses muito baixas, da ordem do valor paramétrico de referência da MCLR para água de consumo humano. Salientam, portanto, o interesse de avaliar a capacidade da MCLR induzir a proliferação celular no rim *in vivo* e alertam para a importância de rever o valor paramétrico de

referência / valor-guia no sentido de contemplar eventuais efeitos cancerigénicos, não só no fígado como também noutros órgãos.

A pesquisa de efeitos genotóxicos da MCLR tem gerado alguns resultados contraditórios e inconclusivos. A hipótese da MCLR formar aductos com a molécula de ADN e ser, assim, um agente directamente mutagénico parece pouco provável. (Bouaïcha et al., 2005). Por sua vez, a capacidade da MCLR induzir quebras de cadeia dupla no ADN (avaliada pelo ensaio do Cometa) tem também originado interpretações distintas. Por um lado, alguns autores consideram que essas lesões decorrem da indução de stress oxidativo (Žegura et al., 2003, 2004, 2006, 2008; Nong et al., 2007). Por outro lado, outros autores consideram que as quebras de ADN observadas são um reflexo da fragmentação de ADN durante a apoptose e não de efeitos genotóxicos (Rao et al., 1998; Lankoff et al., 2004). Embora no presente trabalho não tenha sido avaliado o efeito da MCLR ao nível do stress oxidativo, alguns dos efeitos celulares descritos no capítulo 4 (danos mitocondriais e apoptose) sugerem o envolvimento de espécies reactivas de oxigénio na toxicidade da MCLR na linha celular Vero-E6. No entanto, os resultados negativos obtidos no teste do Cometa (capítulo 5) discordam com a hipótese de, nas concentrações testadas, a MCLR induzir danos oxidativos no ADN, detectáveis como quebras de ADN no referido ensaio, nos modelos celulares Vero-E6 e HepG2.

A análise de anomalias cromossómicas estruturais não evidenciou qualquer efeito associado à MCLR (Lankoff et al., 2004, 2006a). No entanto, o aumento da frequência de micronúcleos nas células Vero-E6 e HepG2 (capítulos 2 e 5) é indicativo da capacidade da MCLR induzir danos ao nível cromossómico. Estes resultados, juntamente com os de outros autores, sugerem que esse efeito não ocorre através de um mecanismo de clastogénese (quebra cromossómica), mas possivelmente de aneugénese (perda cromossómica), quer em células hepáticas quer em células renais. Esta hipótese é compatível com a observação de que a MCLR induz a desorganização de elementos do citosqueleto nas células Vero (capítulo 4), uma vez que a aneugénese pode resultar de danos nos microtúbulos do fuso mitótico (Tournebize et al., 1997; Parry et al., 2002). A aneugénese pode ter como consequência a aneuploidia (alteração do número diplóide de cromossomas), que tem sido associada ao desenvolvimento de cancro no Homem (Kirsch-Volders et al., 2002), podendo assim contribuir para a eventual cancerigenicidade da MCLR.

A distinção entre actividade aneugénica e clastogénica pode ser um aspecto importante para a avaliação do risco, um vez que os agentes aneugénicos,

contrariamente aos clastogénicos, caracterizam-se por uma relação dose-resposta que permite estabelecer um limiar de genotoxicidade, abaixo do qual não decorrem quaisquer riscos para a saúde (Kirsch-Volders et al., 2002; Iarmarcovai et al., 2006).

Apesar das lacunas que ainda permanecem acerca da genotoxicidade das microcistinas, a publicação crescente e recente de estudos sobre esta matéria salienta, novamente, a importância do estudo aprofundado acerca do potencial cancerigénico das microcistinas e da revisão da legislação sobre o teor destas cianotoxinas em água destinada à utilização humana.

Numa exercício de reavaliação do valor-guia das microcistinas, de forma a contemplar o seu efeito de promoção tumoral, Duy et al. (2000) propõe o valor de 0.07 $\mu g.L^{-1}$ (0.07 nM). Por outro lado, um estudo epidemiológico (Zhou et al., 2002) revelou que a ingestão de água contendo 50 $ng.L^{-1}$ (0.05 nM) de microcistinas aumenta em cerca de 8 vezes o risco de cancro colorectal. Na linha celular Vero-E6, a MCLR estimulou a proliferação celular na gama de 1-10 nM. Estes dados indicam que o actual valor guia (1 nM) poderá estar, de facto, acima de um valor de segurança que salvaguarde efeitos potencialmente cancerigénicos.

De salientar, ainda, que a exposição a doses baixas de MCLR poderá corresponder à situação mais realista ao nível renal. Não sendo um órgão onde a MCLR se acumule significativamente, o rim é responsável, em parte, pela eliminação da toxina. Assim, e embora não esteja devidamente caracterizada a toxicocinética da MCLR, poderá presumir-se que a dose interna de MCLR neste órgão seja baixa. Além disso, e de acordo com os resultados discutidos no capítulo 6, poderá supor-se que a dose biológica efectiva para induzir a progressão no ciclo celular seja igualmente baixa. Neste sentido, e não descurando os eventuais efeitos nefrotóxicos (agudos) da MCLR, o estudo dos seus efeitos crónicos assume particular importância.

Um outro aspecto importante relativamente à avaliação do risco é a eventual exposição simultânea a várias cianotoxinas. Os *blooms* cianobacterianos são frequentemente compostos por espécies ou estirpes distintas, que produzem diferentes toxinas (Chorus et al., 2000; Duy et al, 2000). Para além da MCLR também a variante MCRR (Žegura et al., 2002), a nodularina (Lankoff et al., 2006b) e a cilindrospermopsina (Humpage et al., 2000b), são potenciais genotoxinas, desconhecendo-se, ainda, se apresentam entre si efeitos genotóxicos antagonistas ou sinergisticos.

3. Fosfatases proteicas PP1/PP2A e stress oxidativo: os pontos centrais na toxicidade da MCLR no modelo celular Vero-E6?

A inibição das fosfatases proteicas PP1 e PP2A tem sido considerada como o mecanismo responsável pela hepatotoxicidade aguda da MCLR (Yoshizawa et al., 1990). Foi também sugerido que é possivelmente um mecanismo que medeia a actividade de promoção tumoral associada à MCLR (Gehringer, 2004). As fosfatases PP1 e PP2A têm um carácter ubíquo nas células de mamífero, têm um papel fulcral na regulação de inúmeros processos metabólicos e fisiológicos, bem como na manutenção da homeostase celular (Dawson e Holmes, 1999). Embora no presente trabalho não tenha sido avaliado o efeito da MCLR na actividade das fosfatases PP1 e PP2A, propõe-se que estas desempenhem um papel central na toxicidade da MCLR no modelo celular Vero-E6, tal como tem sido descrito para células hepáticas. De facto, efeitos tão distintos como as alterações da estrutura do citosqueleto (Toivola e Eriksson, 1999), o distúrbio do fuso mitótico e a consequente indução de micronúcleos por aneugénese (Tournebize et al., 1997), bem como a actividade das cinases ERK1/2 (Junttila et al., 2008) poderão ser, efectivamente, desencadeados pelo desequilíbrio entre os estados de fosforilação e desfosforilação e, portanto, dependentes do *turnover* entre cinases e fosfatases.

Por outro lado, o stress oxidativo tem sido também considerado com um processo central da toxicidade da MCLR em hepatócitos. A indução de espécies reactivas de oxigénio pela MCLR, resultante da depleção dos níveis de GSH, tem sido associada a efeitos tão diversos como genotoxicidade (Žegura et al., 2003, 2004, 2006, 2008) e apoptose (Ding e Ong, 2003). Tal como se referiu anteriormente, o stress oxidativo não parece ter um papel preponderante na genotoxicidade induzida pela MCLR nas linhas celulares HepG2 e Vero-E6 (capítulo 5), embora sejam ainda necessários mais estudos para clarificar este aspecto. No entanto, a maioria dos efeitos descritos nas células Vero-E6 ao nível dos organelos celulares (capítulo 4) e da citotoxicidade (capítulo 3), associados a autofagia, apoptose e necrose, resultaram, possivelmente, do desequilíbrio entre a indução de espécies reactivas de oxigénio e a capacidade de defesa antioxidante. Na figura 3 esquematizam-se os efeitos induzidos pela MCLR no modelo celular Vero-E6 e propõe-se alguns mecanismos eventualmente envolvidos na toxicidade da MCLR.

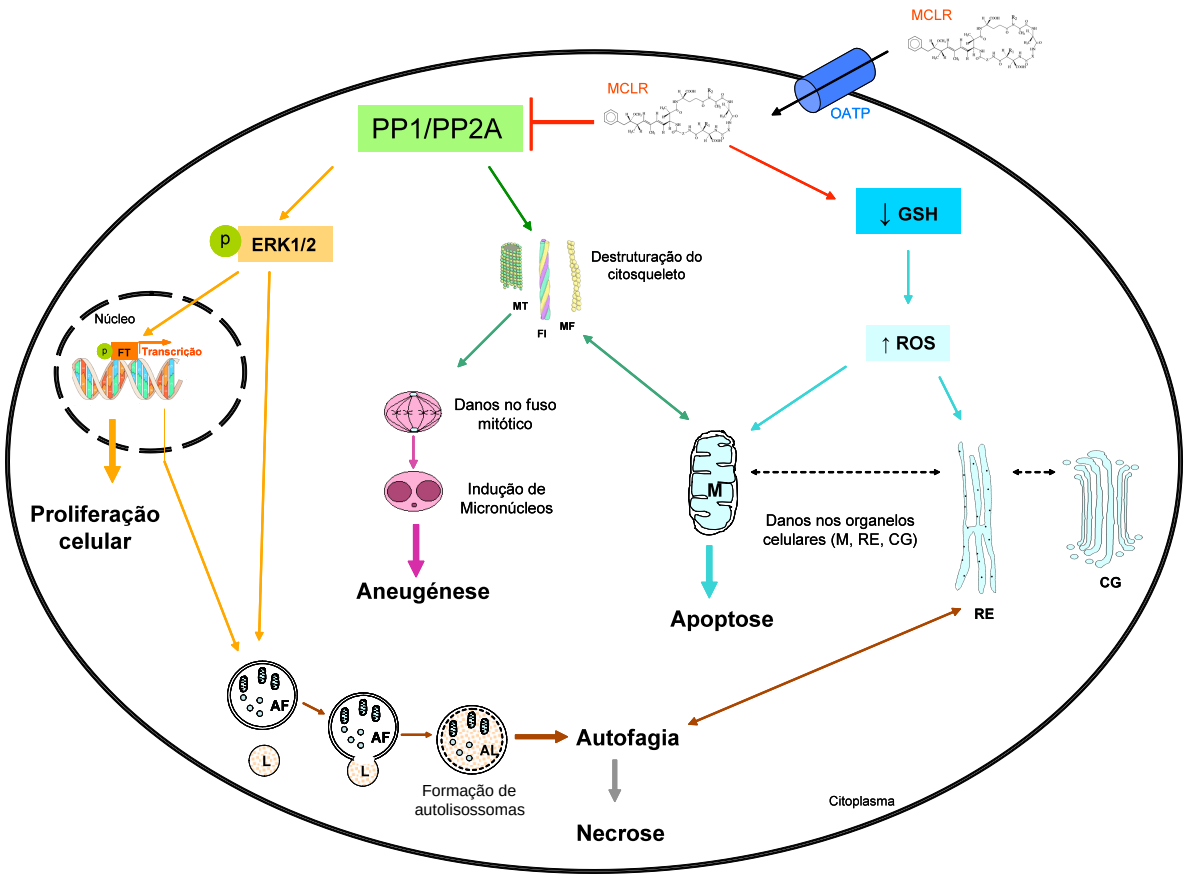


Figura 3. Efeitos e mecanismos de toxicidade da MCLR no modelo celular Vero-E6.

Legenda: **AF** (autofagossoma); **AL** (autolisossoma); **CG** (complexo de Golgi); **ERK1/2** (*extracellular-signal-regulated kinase*); **FI** (filamentos intermédios); **FT** (factores de transcrição); **GSH** (forma reduzida de glutationo); **L** (lisossoma); **M** (mitocôndria); **MF** (microfilamentos); **MT** (microtúbulos); **OATP** (transportadores polipeptídicos dos aniões orgânicos); **P** (grupo fosfato); **PP1/PP2A** (fosfatases proteicas do tipo 1 e 2A); **RE** (retículo endoplasmático); **ROS** (espécies reactivas de oxigénio). ⊥ inibição.

Conclusões e perspectivas

Enumeram-se, seguidamente, as principais conclusões do trabalho apresentado na presente dissertação:

1- A MCLR induz vários tipos de efeitos tóxicos na linha celular renal Vero-E6, em função da concentração;

2- A MCLR tem actividade genotóxica (indução de micronúcleos) através, presumivelmente, de um mecanismo aneugénico;

3- Destaca-se a capacidade da MCLR induzir a proliferação celular (através da activação da via de sinalização ERK1/2) para concentrações de toxina na ordem de grandeza do valor paramétrico de referência / valor-guia para água de consumo humano;

4- A linha celular Vero-E6 parece constituir um modelo celular adequado ao estudo de efeitos da MCLR ao nível renal, designadamente efeitos potencialmente cancerigénicos.

Ficaram por esclarecer algumas questões que poderão constituir tema de pesquisa futura. Sugere-se, assim, como trabalho de continuidade ao que aqui foi apresentado:

1- Esclarecer qual o mecanismo de genotoxicidade das microcistinas, atendendo à importância da distinção entre actividade aneugénica e clastogénica. Esta distinção poderá ser conseguida através da caracterização do conteúdo dos micronúcleos por aplicação da técnica hibridação in situ de fluorescência de (FISH) recorrendo a uma sonda pan-centromérica;

2- Estudar os mecanismos de acção da MCLR a montante das cinases ERK1/2 de forma a elucidar o efeito da MCLR na via de sinalização Ras-Raf-MEK-ERK;

3- Avaliar a capacidade da MCLR induzir efeitos no rim, em particular efeitos cancerigénicos. Um estudo preliminar está já a ser conduzido em murganhos (no Laboratório de Toxicologia Genética / INSA) com o objectivo de avaliar a actividade genotóxica da MCLR *in vivo*.

Referências bibliográficas

Andrinolo, D., Sedan, D., Telese, L., Aura, C., Masera, S., Giannuzzi, L., Marra, C.A., Alaniz, M.J.T. 2008. Hepatic recovery after damage produced by sub-chronic intoxication with the cyanotoxin microcystin LR. Toxicon 51: 457-467.

Bouaïcha, N.,. Maatouk, I., Plessis, M.J., Périn, F. 2005. Genotoxic potential of microcystin-LR and nodularin *in vitro* in primary cultured rat hepatocytes and *in vivo* rat liver. Environ. Toxicol. 20: 341-347.

Chorus, I., Falconer, I.R., Salas, H.J., Bartram, J. 2000. Health risks caused by freshwater cyanobacteria in recreational waters. J. Toxicol. Environ. Health, Part B 3: 323-347.

Dawson, J.F., Holmes, C.F.B. 1999. Molecular mechanisms underlying inhibition of protein phosphatases by marine toxins. Frontiers in Bioscience 4, d646-658.

Ding, W.X., Ong, C.N. 2003. Role of oxidative stress and mitochondrial changes in cyanobacteria-induced apoptosis and hepatotoxicity, FEMS Microbio. Lett. 10880: 1-7.

Duy, T.N., Lam, P.K.S., Shaw, G.R., Connell, D.W. 2000. Toxicology and risk assessment of freshwater cyanobacterial (blue-green algae) toxins in water. Rev. Environ. Contam. Toxicol. 163: 113-186.

Falconer, I.R. 1991. Tumour promotion and liver injury caused by oral consumption of cyanobacteria, Environ. Toxicol. Water Qual. 6: 177-184.

Gehringer, M.M. 2004. Microcystin-LR and okadaic acid-induced cellular effects: a dualistic response. FEBS Letters 557, 1-8.

Humpage, A.R., Hardy, S.J., Moore, E.J., Froscio, S.M., Falconer, I.R. 2000a. Microcystins (cyanobacterial toxins) in drinking water enhance the growth of aberrant crypt foci in the mouse colon. J. Toxicol Environm Health A 61: 155-65.

Humpage, A.R., Fenech, M., Thomas, P., Falconer, I.R. 2000b. Micronucleous induction and chromosome loss in transformed human white cells indicate clastogenic and aneugenic action of the cyanobacterial toxin, cylindrospermopsin. Mutat. Res. 472: 155-161.

Iarmarcovai, G., Botta, A., Orsière, T.2006. Number of centromeric signals in micronuclei and mechansims of aneuploidy. Toxicology Letters 166: 1-10.

Ito, E., Kondo, F., Terao, K., Harada, K.I. 1997. Neoplastic nodular formation in mouse liver induced by repeated intraperitoneal injection of microcystin-LR, Toxicon 35: 1453-1457.

Junttila, M.R., Li, S.P., Westermarck, J. 2008. Phosphatase-mediated crosstalk between MAPK signalling pathways in the regulation of cell survival. FASEB J. 22: 954-965.

Kirsch-Volders, M., Vanhauwaert, A, De Boeck, M., Decordier, I. 2002. Importance of detecting numerical versus structural chromosome aberrations. Mut. Res. 504 : 137-148.

Komatsu, M., Furukawa, T., Ikeda, R., Takumi, S., Nong, Q., Aoyama, K., Akiyama, S.I., Keppler, D., Takeuchi, T. 2007. Involvment of mitogen-activated protein kinase signalling pathways in microcystins-LR-induced apoptosis after its selective uptake mediated by OATP1B1 and OATP1B3. ToxicolSci. 97: 407-416.

Lankoff, A., Krzowski, L., Glab, J., Banasik, A., Lisowska, H., Kuszwski, T., Gozdz, S., Wojcik, A. 2004. DNA damage and repair in human peripheral blood lynphocytes following treatment with microcystin-LR. Mut. Res. 559: 131-142.

Lankoff, A., Bialczyki, J., Dziga, D., Carmichael, W.W., Gradzka, I., Lisowska, H., Kuszewski, T., Gozdz, S., Piorun, I., Wojcik, A. 2006a. The repair of gamma-radiation-induced DNA damage is inhibited by microcystin-LR, the PP1 and PP2A phosphatase inhibitor. Mutagenesis 21:) 83-90.

Lankoff, A., Wojcik, A., Fessard, V., Meriluoto, J. 2006b. Nodularin-induced genotoxicity following oxidative DNA damage and aneuploidy in HepG2 cells. Toxicology Letters 164: 239-248.

Milutinović, A., Sedmark, B., Horvat-ŽnidarŠić, I., Šuput, D. 2002. Renal injuries induced by chronic intoxication with microcystins. Cell. Mol. Biol. Lett. 7: 139-141.

Milutinović, A., Živin, M., Zorc-Plesković, R., Sedmark, B., Šuput, D. 2003. Nephrotoxic effects of chronic administration of microcystins-LR and –YR. Toxicon 42: 281-288.

Moreno, I., Pichardo, S., Jos, A., Gómez-Amores, L., Mate, A., Vazquez, C.M., Cameán, A.M. 2005. Antioxidant enzyme activity and lipid peroxidation in liver and kidney of rats exposed to microcystin-LR administered intraperitoneally. Toxicon 45: 395-402.

Nishiwaki-Matsushima, R., Ohta, T., Nishiwaki, S., Suganuma, M., Kohyama, K., Ishikawa, T., Carmichael, W.W., Fujiki, H. 1992. Liver tumor promotion by the cyanobacterial cyclic peptide toxin microcystin-LR. J. Cancer Res. Clin. Oncol. 118: 420–424.

Nobre, A.C.L., Jorge, M.C.M., Menezes, D.B., Fonteles, M.C., Monteiro, H.S.A. 1999. Effects of microcystin-LR in isolated perfused rat kidney. Brazilian Journal of Medical and Biological Research 32: 985-988.

Nong, Q., Komatsu, M., Izumo, K., Indo, H.P., Xu, B., Aoyama, K., Majima, H.J., Horiuchi, M., Morimoto, K., Takeuchi, T. 2007. Involvment of reactive oxigen species in microcystin-LR-induced cytogenotoxicity. Free Radical Res. 4: 1326-1337.

Parry, E.M., Parry, J.M., Corso, C., Doherty, A., Haddad, F., Hermine, T.F., Johnson, G., Kayani, M., Quick, E., Warr, T., Williamson, J.2002. Detection and characterization of mechanisms of action of aneugenic chemicals. Mutagenesis 17: 509-521.

Rao, P.V., Bhattacharya, R., Parida, M., Jana, A., Bhaskar, A. 1998. Freshwater cyanobacterium Microcystis aeruginosa (UTEX 2385) induced DNA damage *in vivo* and *in vitro*. Environ. Toxicol. Pharmacol. 5: 1-6.

Robinson N.A., Pace J.G., Matson C.F., Miura G.A., Lawrence W.B. 1990. Tissue distribution, excretion and hepatic biotransformation of microcystin-LR in mice. J. Pharmacol.Exp. Ther. 256:176-182.

Toivola, D.M., Eriksson, J.E. 1999. Toxins affecting cell signaling and alteration of cytoskeletal structure. Toxicol. *In Vitro* 13: 521-530.

Tournebize, R., Andersen, S., Verde, F., Dorée, M., Karsenti, E., Hyman, A. 1997. Distinct roles of PP1 and PP2A-like phosphatases in control of microtubule dynamics during mitosis. The Embo journal 16: 5537-5549.

W.H.O. 1998. Guidelines for drinking water quality, Second Edition, Addendum to Volume 2, Health Criteria and other supporting information. World Health Organization, Geneva.

Yoshizawa, I., Matsushima, R., Watanabe, M.F., Harada, H., Ichihara, A., Carmichael, W.W., Fujiki, H. 1990. Inhibition of protein phosphatases by microcystis and nodularin associated with hepatotoxicity. J. Cancer Res. Clin. Oncol. 116: 609-614.

Žegura, B., Filipič, M., Šuput, D., Lah, T., Sedmak. B. 2002. *In vitro* genotoxicity of microcystin-RR on primary cultured hepatocytes and HepG2 cell line detected by Comet assay. Radiol. Oncol. 36: 159-61.

Žegura, B., Sedmak, B., Filipič, M. 2003. Microcystin-LR induces oxidative DNA damage in human hepatoma cell line HepG2. Toxicon 41: 41-48.

Žegura, B., Lah, T., Filipič, M. 2004. The role of reactive oxygen species in microcystin-LR-induced DNA damage. Toxicology 200: 59-68.

Žegura, B., Lah, T., Filipič, M. 2006. Alteration of intracellular GSH levels and its role in microcystin-LR-induced DNA damage in human hepatoma HepG2 cells. Mut. Res. 611: 25-33.

Žegura, B., Volčič, M., Lah, T., Filipič, M. 2008. Different sensitivities of human colon adenocarcinoma (CaCo-2), astrocytoma (IPDDC-A2) and lymphoblastoid (NCNC) cell lines to microcystin-LR induced reactive oxygen species and DNA damage. Toxicon 52: 518-525.

Zhou, L., Yu, H., Chen K. 2002. Relationship between microcystin in drinking water and colorectal cancer. Biomed Environ. Sci.15: 166-71.

Zhu, Y., Zhong, X., Zheng, S., Ge, Z., Du, Q., Zhang, S. 2005. Transformation of immortalized colorectal crypt cells by microcystin involving constitutive activation of Akt and MAPK cascade. Carcinogenesis 26: 1207-1214.