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Comparative study of the cytotoxic effects of microcystin-LR in mammalian cell lines: Vero, HepG2, Caco2 and MDCK.

Carina Alexandra Gomes Menezes

Mestrado em Biologia Humana e Ambiente

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COMPARATIVE STUDY OF THE CYTOTOXIC EFFECTS OF MICROCYSTIN-LR IN MAMMALIAN CELL LINES: VERO, HEPG2, CACO2 AND MDCK.

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Mestrado em Biologia Humana e Ambiente

2009

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LIST OF ABBREVIATIONS

AbDil – Antibody Dilution Solution

ADDA-(2S,35',8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-(4E),(6E)-decadienoic acid

AO – Acridine Orange

ATCC – American Type Culture Collection

Bcl-2 – anti-apoptotic protein

CaCo2 – Human colon adenocarcinoma cell line

CBS – Cytoskeleton Buffer with sucrose

CCD – Cooled camera device

DAPI – 4'-6-diamidino-2-phenylindole

DMEM – Dulbecco´s Modified Eagle Medium

DMSO – Dimethyl sulfoxide

DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures)

EDTA – Ethylene Diamine Tetraacetic acid

ECL – Enhanced Chemical Luminescence

ER – Endoplasmic reticulum

FBS – Fetal Bovine Serum

GRP94 – Glucose-regulated protein 94

GSH - Glutatione

HepG2 – Human hepatoma cell line

HPLC – High Pressure Liquid Chromatography

INSA - Instituto Nacional de Saúde (National Health Institute)

ISO – International Organization for Standardization

LBE – Laboratory of Biology and Ecotoxicology, National Health Institute Dr. Ricardo Jorge

LC3B – Light chain 3B

LC3B-I – Light chain 3B, form 1 (cytosolic)

LC3B-II – Light chain 3B, form 2 (membranous)

LD_{50} – Lethal dose to 50% of the treated animals

LDH – Lactate dehydrogenase

LMECYA 110– *Microcystis aeruginosa* toxin producer strain kept in culture in LBE

LMECYA 127 - *M. aeruginosa* non-toxin producer strain kept in culture in LBE

LMP – Lysosome membrane potential

MDCK – Dog kidney-derived cell line

MCLR – Microcystin-LR

MEM – Modified Eagle Medium

MMP – Mitochondrial membrane potential

MPT – Mitochondrial permeability transition state

MTT – 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide

NR – Neutral Red

OATP – Organic anion transporting polypeptide

PBS – Phosphate buffered saline

PBS/T – Phosphate buffered saline with 0,02% Tween

PFA – Paraformaldehyde

PP1 – Protein Ser/Thr Phosphatase type 1

PP2A - Protein Ser/Thr Phosphatase type 2A

PVDF – Polyvinylidene Fluoride

Rh-123 – Rhodamine-123

ROS – Reactive oxygen species

rt – room temperature

SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis

TEM – Transmission Electron Microscopy

TBS - Tris-buffered saline

TBS/T – Tris-buffered saline with 0,02% Tween

Vero – African green monkey *Cercopithecus aethiops* kidney-derived cell line

WHO – World Health Organization

ACKNOWLEDGMENTS /AGRADECIMENTOS

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ABSTRACT

Microcystin-LR (MCLR) is a natural occurring freshwater cyanotoxin, recognized as one of the most toxic microcystin variants. It is thought to be responsible for cases of livestock and human intoxication due to consumption of toxic cyanobacteria-contaminated water. Although considered a hepatotoxin, MCLR also targets other organs such as the kidneys and intestines. In spite the cellular mechanisms associated with the toxicity of MCLR are still unclear, a previous work in a monkey kidney cell line suggested that the endoplasmic reticulum was an early target of MCLR toxicity and that autophagy was triggered as a cell defense mechanism at subcytotoxic concentrations of MCLR.

In the present work, cytotoxic, morphological and ultrastructural effects of MCLR were compared in HepG2 (human liver), Vero (monkey kidney), MDCK (dog kidney) and Caco2 (human intestine) cell lines. MCLR induced a concentration-dependent decrease in cell viability by the NR assay in all cell lines, with HepG2 and Vero showing the lowest cytotoxic thresholds of 25 and 50 µM MCLR, respectively. In these cells, MCLR exposure induced lysosomal damages previously to mitochondrial disruption, reinforcing the role of lysosomes in MCLR-induced toxicity. Immunolabelling and ultrastructural visualization of autophagosomes, showed that autophagy was a response transversal to both cell lines, triggered at subcytotoxic MCLR concentrations, confirming its importance as a defense mechanism to early damages inflicted by the toxin. The analysis of GRP94, an ER stress protein, did not undoubtedly demonstrate that MCLR targets the ER. However, together with the ultrastructural data, suggested that in both HepG2 and Vero cells, the ER has a role in autophagy induction. Additionally, in HepG2 cells, GRP94 down-regulation with increasing MCLR concentrations supported the ER role in the triggering of apoptosis. At high toxin concentrations, ultrastructural alterations consistent with apoptosis were observed for all four cell lines, proving that this is a general MCLR-induced mechanism.

RESUMO ALARGADO

As cianobactérias são organismos procariotas com origem há cerca de 2,8 mil milhões de anos e que ainda hoje proliferam em todos os ecossistemas da Terra. O aparecimento de florescências de cianobactérias é um fenómeno potenciado pela acção do homem nos ecossistemas através da eutrofização das massas de águas e que acarreta a potencial libertação de toxinas produzidas por estes organismos (cianotoxinas). Estes fenómenos são mais comuns em sistemas de água doce pelo que a possibilidade de intoxicação por cianotoxinas em águas de consumo ou de recreio é actualmente uma preocupação em Saúde Pública, em particular em regiões com elevada dependência de reservas de água superfícial (e.g.rios, albufeiras, lagos) como é o caso de Portugal .

As microcistinas são as cianotoxinas mais frequentes, sendo detectadas na maior parte das florescências de cianobactérias. São também, as cianotoxinas mais estudadas e melhor caracterizadas em relação à estrutura, actividade e toxicologia. De entre as 70 variantes conhecidas de microcistinas, a microcistina-LR (MCLR) é uma das mais comuns e mais tóxicas. Alguns casos humanos de intoxicação aguda por microcistinas foram já descritos e apresentaram como principais manifestações clínicas vómitos, diarreia, hemorragia hepática e, nos casos mais graves, a morte dos indivíduos.

Actualmente, a MCLR é reconhecida sobretudo como uma hepatotoxina, uma vez que o seu órgão-alvo principal é o fígado. Devido à natureza hidrofílica da MCLR, a sua entrada nas células é mediada pelos transportadores dos ácidos biliares (OATP). Estes estão presentes em grande quantidade no fígado e em menor extensão noutros órgãos como o cérebro, rins e intestinos. Desta forma, a maioria dos estudos sobre os efeitos da MCLR têm sido realizados principalmente em células hepáticas *in vivo* ou *in vitro.* No entanto, estudos recentes têm demonstrado efeitos tóxicos da MCLR em células renais e de intestino.

Um dos mecanismos de toxicidade da MCLR melhor documentados é a inibição das fosfatases proteicas 1 e 2A. Estas são importantes reguladoras de proteínas implicadas em inúmeros processos celulares tais como o metabolismo, a manutenção do citosqueleto e a divisão celular. O efeito inibitório da MCLR sobre as fosfatases PP1 e PP2A conduz à despolimerização e agregação dos componentes do citosqueleto, levando ao colapso da estrutura dos hepatócitos.

Por outro lado, grande parte dos artigos científicos publicados descreve também a mitocôndria como um organelo-alvo da toxicidade da MCLR, designadamente o seu papel mediador no processo apoptótico induzido pela MCLR. Este processo, por sua vez, parece ser desencadeado pela indução da produção de espécies reactivas de oxigénio pela MCLR. Mais recentemente, outros organelos celulares começaram a ser também identificados como alvos intracelulares da MCLR.

Em trabalhos prévios realizados no Laboratório de Biologia e Ecotoxicologia, INSA com a linha celular renal de macaco verde africano Vero-E6 (Alverca et al., 2009), foi demonstrado que antecedendo o efeito apoptótico, a MCLR induz a autofagia e que estes dois processos envolvem não só o citosqueleto e as mitocôndrias, como também outros organelos celulares tais como os lisossomas e o retículo endoplasmático. O presente estudo, surge na sequência deste trabalho e procurou estudar se os efeitos observados na linha renal Vero-E6 também ocorrem noutros tipos celulares. Neste contexto, os efeitos citotóxicos, morfológicos e ultraestruturais da MCLR foram comparados em quatro linhas celulares: 1) HepG2 (hepatoma humano); 2) Vero-E6 (rim de macaco); 3) MDCK (rim de cão); 4) CaCo2 (adenocarcinoma do cólon humano). Estas linhas celulares permanentes representam, neste estudo, os vários órgãos alvo da MCLR.

A viabilidade celular dos diferentes tipos celulares expostos à MCLR foi avaliada através do teste de citotoxicidade do Vermelho Neutro, que se baseia na integridade dos lisossomas. Em todas as linhas celulares estudadas houve um decréscimo da viabilidade celular de uma forma dependente da concentração de toxina, embora estas tenham apresentado sensibilidades diferentes. O decréscimo de viabilidade foi significativo aos 25 µM de MCLR para as células HepG2, aos 50 µM, para as células Vero e aos 100 µM para as células MDCK e CaCo2. Estes dados foram consistentes com as alterações ultraestruturais observadas, embora nas células MDCK se tenham observado alterações estruturais nas mitocôndrias a concentrações de toxina mais baixas (25 µM de MCLR). Os limiares de citotoxicidade obtidos reflectem a situação de intoxicação com MCLR *in vivo,* em que o fígado é o orgão mais afectado, seguido dos rins e intestinos. Assim, é também demonstrada a boa representabilidade destas linhas celulares enquanto modelos de órgãos-alvo da MCLR.

Face à maior sensibilidade das células HepG2 e Vero à MCLR, apenas estas duas linhas celulares foram usadas para a avaliação dos efeitos da MCLR ao nível dos organelos

e processos celulares. Foram efectuadas marcações específicas de lisossomas, mitocôndrias e filamentos de actina através do uso de fluorocromos específicos (laranja de acridine, rhodamina-123 e faloidina) e marcação com anticorpos para uma proteína do retículo endoplasmático (GRP94) e para uma proteína dos autofagossomas (LC3B). Os resultados obtidos nas duas linhas celulares mostram que a perturbação dos organelos celulares é dependente da concentração de MCLR. De facto, e para ambas as linhas celulares, concentrações relativamente baixas de toxina (12 µM MCLR) induziram a diminuição do número de lisosomas, mas o aumento do seu tamanho, enquanto que a concentrações mais elevadas (25 µM) a MCLR induziu a ruptura destes organelos. As alterações ao nível mitocondrial e nos filamentos de actina foram observadas de forma generalizada, apenas a concentrações de toxina superiores (50 µM MCLR). Assim, nestas linhas celulares, e ao contrário do que é tradicionalmente descrito, os danos nos lisossomas antecedem os das mitocôndrias, sugerindo que terão um papel importante na acção tóxica da MCLR.

A proteína LC3B associa-se à membrana dos autofagossomas, sendo um bom marcador da autofagia. A imunolocalização desta proteína e a análise ultrastructural, demonstrou que existe indução de autofagia a concentrações baixas de toxina (6 e 12 µM MCLR) para as duas linhas celulares confirmando os resultados obtidos em estudos prévios para as células Vero. No entanto, esta resposta autofágica foi mais pronunciada nas células HepG2 do que nas células Vero. De facto, a análise ultrastrutural e de fluorescência, permitiram identificar inúmeros autofagossomas de grande tamanho nas células HepG2. Este aspecto estará provavelmente relacionado com as funções de destoxificação fundamentais nas células hepáticas. Embora a autofagia pareça ser um importante mecanismo de defesa contra danos celulares precoces induzidos pela MCLR, como indicam os resultados das células HepG2 e Vero, não é um processo geral de resposta celular à toxina, já que no estudo ultrastrutural não foi detectada nas linhas celulares MDCK e CaCo2.

A proteína GRP94 existe no retículo endoplasmático de células fisiologicamente normais. No entanto, está também envolvida em processos de reparação associados com o stress do retículo e apresenta propriedades anti-apoptóticas. Os resultados da imunolocalização desta proteína revelaram que o seu padrão de distribuição é dependente da linha celular. Por outro lado, também a expressão da GRP94 foi afectada diferencialmente nas duas linhas celulares estudadas. Assim, por *western blot* verificou-se

que a expressão desta proteína diminui com o aumento da concentração de MCLR indiciando que na linha hepática a activação de vias apoptóticas parece ser mediada pelo retículo endoplasmático. Por outro lado, na linha celular renal a expressão da proteína GRP94 manteve-se inalterada, independentemente da concentração de MCLR. Isto sugere que nas células Vero a apoptose induzida pela MCLR não será activada pelo retículo endoplasmático, mas sim por outros organelos como sejam as mitocôndrias ou os lisossomas. A concentrações elevadas de toxina, observaram-se alterações ultrastruturais consistentes com a apoptose nas quatro linhas celulares em estudo, comprovando que este é um mecanismo geral de resposta celular à MCLR.

KEYWORDS/PALAVRAS-CHAVE

Keywords

Microcystin-LR

Mammalian cell lines

Cytotoxicity

Cellular organelles

Autophagy

Apoptosis

Ultrastructure

Palavras-chave

Microcistina-LR

Linhas celulares de mamífero

Citotoxicidade

Organelos celulares

Autofagia

Apoptose

Ultrastrutura

INTRODUCTION

1. Cyanobacteria

1.1. Characterization

The Cyanobacteria or Cyanoprokaryota, commonly known as blue-green algae, are a group of ancient photosynthetic organisms present on Earth since 2.7 billion years ago (Badger and Price, 2003). This group is divided into the four orders Chroococcales, Oscillatoriales, Nostocales and Stigonematales, including 2000 species grouped in 150 genera (Chorus and Bartram, 1999). These organisms can be found in a wide variety of environmental conditions such as soil, rocks, desert sand, volcanic ash, cold or hot water springs and salt, brackish or freshwaters, in different forms such as primitive single-celled, colonial or filamentous (Chorus and Bartram, 1999). They are characterized by the absence of a nucleus and membrane-bound organelles and have a cell wall composed of peptidoglycan and lipopolysaccharides (Oberholster et al., 2004). These aerobic photoautotrophic organisms possess chlorophyll a and accessory pigments (phicobilipigments such as phycocyanin, allophycocyanin and phycoerythrin), acquiring blue-green to violet-red cell colors and the ability to perform water-splitting, oxygenic photosynthesis (Chorus and Bartram, 1999; Chorus et al., 2000).

The successful adaptation of several planktonic forms to the aquatic environment may be explained by the presence of cellular specializations in some species. Heterocysts are cells specialized in nitrogen fixation, while akinetes are resistance cysts that ensure survival under unfavorable environmental conditions. Additionally, intracellular gas vesicles named aerotopes, enable movement along the water column favoring better light and oxygen conditions. This ability is termed buoyancy and is achieved by means of a balance between carbohydrate and gas volume content in the aerotopes (Chorus et al., 2000).

1.2. Blooms

The occurrence of favorable freshwater environmental conditions may trigger the development of cyanobacterial blooms. These are massive developments of cyanobacteria, usually characterized by a slimy scum at the water surface (Falconer, 2005). Blooms generally occur in late summer or early fall when the temperatures are usually between 15 and 30°C. Also, eutrophic or hyper-eutrophic bodies of water containing adequate levels of essential inorganic nutrients such as nitrogen and phosphorus and pH levels between 6 and 9 may potentiate bloom occurrence providing optimal bloom conditions (WHO, 1998). A large number of blooms have been reported worldwide over the years (Chorus and Bartram, 1999; Falconer, 1999; Chorus et al., 2000; Figueiredo et al., 2004; Galvão et al., 2008) accompanying the increasing water alterations, such as eutrophication, consequence of

Table 1 - Relevant dates and events in toxic cyanobacteria history (Adapted from Carmichael, 2002).

Date	Event
1878	First reported toxic surface bloom in Australia (Francis, 1878)
1958-64	Isolation and culture of toxic cyanobacterial clones
1964,1968	International conference: • Algae and Man, 1964; • Algae and the Environment, 1968
1971	Establishment of the Estela Sousa e Silva Algal Culture Collection in the Laboratory of Biology and Ecotoxicology, National Health Institute in Lisbon, Portugal (Paulino et al., 2009)
1971-80	Papers by a number of authors on: • world-wide occurrence of toxic *M. aeruginosa*; • *Aphanizomenon. flos-aquae* neurotoxin production; • gastroenteritis outbreaks in human due to cyanobacteria; • characterization of LSP endotoxin from cyanobacteria
1981-86	• Continuing cases of animal deaths; human gastroenteritis and contact irritation • Research programs on toxic blue-green algae in Norway, Scotland, Germany, Finland, S. Africa, Australia, Japan, U.S.S.R., China and India • Structure of microcystin determined by D. Botes and colleagues (CSIRO, Pretoria, RSA) • Inclusion of toxic cyanobacterial research in several international symposia, especially IUPAC and IST
1987-92	• Continuing cases of animal deaths, human contact irritation and gastroenteritis • Association of cyanotoxins in human drinking water supplies with hepatoenteritis and primary liver cancer • Structure determination of numerous microcystins and identification of nodularin • Structure determination of anatoxin-a(s) and cylindrospermopsin
1993	First report of a toxic bloom in Portugal (Rio Douro in1989; Vasconcelos, 1993).
1993-94	Organization of International Symposia in Bath, United Kingdom, and a workshop in Adelaide, Australia. First exclusive cyanotoxin conference.
1996	• First confirmed human fatalities: cyanotoxins, including microcystins and cylindrospermopsin; Caruaru, Brazil • In Portugal, a monitoring program for cyanobacteria and cyanotoxins in freshwater reservoirs is proposed by the Health Department (Direcção Geral da Saúde) and maintained by some laboratories till the present.
1998	US EPA lists cyanobacteria and cyanotoxins as a research priority on the drinking water candidate contaminant list (Federal Register March 1998)
1999	Establishment of a guidance value for MCLR in drinking waters of 1 µg/L by the WHO.
2000	Number of peer reviewed publications on toxic cyanobacteria exceeds 2200
2007	Guidance value of 1 µg/L MCLR in drinking waters adopted as parametric value in Portuguese law.

human activity. This demonstrates the cyanobacterial remarkable capacity of adaptation to environmental modifications.

So far, as many as 40 species of cyanobacteria are known to be toxin producers. Therefore bloom formation is often accompanied by the release of toxins when toxic cyanobacterial species are present (Carmichael, 1992; Chorus and Bartram, 1999; Falconer, 2005).

Table 1 summarizes some relevant dates concerning toxic cyanobacteria history.

2. Cyanotoxins

Cyanotoxins are secondary metabolites produced by freshwater cyanobacteria that can be classified functionally into neurotoxins, hepatotoxins and dermotoxins or accordingly to its chemical structure into alkaloids, cyclic peptides and lipopolysaccharides (Carmichael, 1992; Hitzfield et al., 2000; Codd, 2000; Chorus et al., 2000; Oberholster et al., 2004; Falconer, 2005). Cyanotoxins are produced by a variety of cyanobacterial genera/species and have distinct toxicities as summarized in table 2.

Table 2 - Types of cyanotoxins, toxic cyanobacterial genera and acute toxicity (Adapted from Chorus and Bartram, 1999; Chorus et al., 2000; Carmichael, 2002).

Toxin	Cyanobacterial genera producers	LD_{50} (ip, mouse)
Neurotoxins		
Anatoxin-a	*Anabaena, Aphanizomenon*	250 µg/kg
Anatoxin-a(s)	*Anabaena, Oscillatoria (Planktothrix)*	40 µg/kg
Saxitoxins	*Anabaena, Aphanizomenon, Lyngbya, Cylindrospermopsis*	10-30 µg/kg
Hepatotoxins		
Cylindrospermopsin	*Aphanizomenon, Cylindrospermopsis*	200 µg/kg
Microcystins	*Anabaena, Nostoc, Microcystis, Oscillatoria (Planktothrix)*	45-1000 µg/kg
Nodularins	*Nodularia*	30-50 µg/kg
Contact irritant-dermal toxins		
Debromoaplysiatoxin, Lyngbyatoxin,	*Lyngbya* (marine)	-
Aplysiatoxin	*Schizothrix* (marine), *Lyngbya, Oscillatoria (Planktothrix)*	
Lipopolysaccharides (LPS)	All	

Neurotoxins are produced by species of *Anabaena, Aphanizomenon, Oscillatoria* and *Lyngbya* and include the alkaloids anatoxin-a and saxitoxin and also the naturally occurring organophosphate anatoxin-a(s) (Carmichael, 1992). Anatoxin-a is an acetylcholine agonist and anatoxin-a(s) is an inhibitor of acetylcholinesterase and both induce continuous muscle

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stimulation leading to paralysis and death within minutes or hours due to respiratory arrest (Vasconcelos, 2001). Saxitoxin is a sodium ion channels blocker that leads to the inhibition of skeletal muscle and peripheral nerves stimulation with consequent death by respiratory arrest (Vasconcelos, 2001; Carmichael, 1992).

Lipopolysaccharides are components of the cellular wall outer layers of all cyanobacterial cells and may act as dermotoxins producing irritations when in direct contact with the skin (Codd, 2000).

Hepatotoxins include nodularin produced by *Nodularia*, cylindrospermopsin produced by *Cylindrospermopsis* and microcystins produced by *Microcystis, Anabaena, Oscillatoria* and *Nostoc* (Chorus and Bartram, 1999; Codd, 2000). Microcystins are the most frequently occurring cyanotoxins and these cyclic heptapeptides were first isolated from *Microcystis aeruginosa* from which the toxins take their name (Carmichael et al., 1988; Chorus and Bartram, 1999).

Cyanotoxins are produced inside the cyanobacterial cells and the release into the surrounding water occurs mainly during cell senescence, cell death or algicide application. Once in the water, they can persist for several days to weeks, constituting a threat even after the bloom as passed (Duy et al., 2000). However, photochemical breakdown in full sunlight can occur with varying rates. Anatoxins are rapidly degraded while cylindrospermopsins and microcystins are rapidly degraded in the presence of cyanobacterial pigments (Chorus and Bartram, 1999). Additionally, aquatic bacteria commonly found in the environment are also responsible for microcystins degradation and clearance from the environment (Jones et al., 1993; Chorus and Bartram, 1999).

3. Microcystins

3.1. Chemical structure

Microcystins are heptapeptides with a general structure composed of five amino acids, D-Alanine, D-Methylaspartic acid, D-Glutamic acid, N-Methyldehydroalanine and a side chain with the specific ADDA amino acid ((2S,3S',8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-(4E),(6E)-decadienoic acid) (positions 1, 3, 6, 7 and 5 in figure 1A respectively), responsible for the biological activity of these cyclic peptides (Carmichael, 1992). The two variable amino acids are responsible for the multiplicity of microcystins variants (X and Z in positions 2 and 4 in figure 1A). To date 70 microcystin variants are known with different hydrophobic/hydrophilic properties as well as different degrees of toxicity, with a LD_{50} (lethal dose to 50% of the treated animals) in intraperitoneal (ip) administered mice of 45-1000 $\mu g.kg^{-1}$ body weight (Carmichael, 1992; WHO, 1998; Chorus et al., 2000).
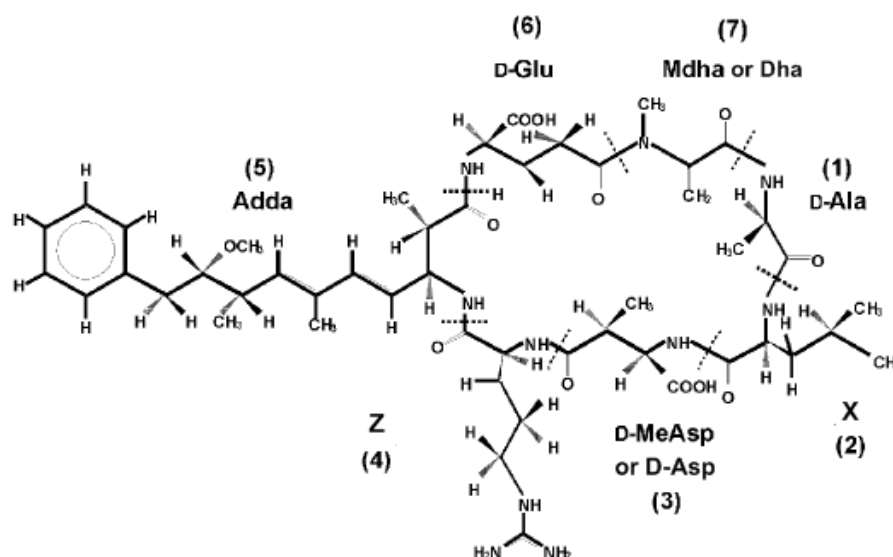
Figure 1 - Chemical structure of microcystins (adapted from Zurawell et al., 2004).

3.2. Occurrence in the environment

Microcystins have attracted attention because of their health effects, wide occurrence and persistence in the environment (Figure 2). From the cyanobacterial samples investigated worldwide, it was estimated that 75% contained toxins, of which microcystin-LR (MCLR) was the most commonly found (WHO, 1998). In Portugal, analysis of cyanobacterial samples collected between 1989 and 1992 concluded that MCLR was the most common microcystin variant and its proportion in each sample ranged from 45.5% to 99.8% of the total microcystin contents (Vasconcelos et al., 1996). In a recent Portuguese study (Galvão et al., 2008), microcystins were detected in 23% of the water samples analyzed (n=51).

Figure 2 - Microcystins worldwide occurrence in freshwater environments (In: Zurawell et al., 2004)

3.3. Human intoxication

Human intoxication can occur through several forms: inhalation of cyanobacterial cells, ingestion of contaminated water from a drinking source or during recreational activities, ingestion of contaminated aquatic organisms, direct skin contact with cyanobacterial blooms and hemodialysis (Chorus et al., 2000; Hitzfield et al. 2000; Benson et al., 2005). The consumption of contaminated food supplies is possible due to the accumulation and transfer along the food chain. In fact, microcystins are known to be transferred through filter-feeding mollusks (such as the mussels), crayfish and fish used for human consumption (Vasconcelos, 1999).

Once ingested, microcystins are transported through the gastrointestinal tract (cell linings of the small intestine) by a specific bile acid transport system to the liver, the primary target of action (Falconer, 1999). The symptoms of acute microcystins intoxication are vomiting and diarrhea, severe liver damage which is characterized by a disruption of liver cell structure, loss of sinusoidal structure, increase in liver weight due to intra-hepatic hemorrhage, heart failure and death (Chorus and Bartram, 1999; Falconer, 2005). Some of these signs can be commonly mistaken with food poisoning, making it difficult to assess microcystins intoxication. When high quantities of toxin are ingested, renal failure may also occur that, in most severe cases, can lead to death (Chorus et al., 2000). One of the most studied and recognized case of human intoxication by cyanotoxins was the Caruaru syndrome which occurred in Brazil in 1996. This incident was caused by the use of cyanotoxins-contaminated water in a dialysis clinic and resulted in 76 deaths, of which 52 were attributed to cyanotoxins (Jochimsen et al., 1998; Charmichael et al., 2001; Azevedo et al., 2002). However, reported cases of livestock and human acute intoxications have multiplied over the past few years (Griffiths and Saker, 2003; Dittman and Wiegand, 2006).

3.4. Microcystin-LR

3.4.1. Chemical properties. Toxin uptake, distribution and elimination.

MCLR is the most extensive studied and characterized microcystin. It is the most common and toxic variant, exhibiting an i.p. LD_{50} of 50 $\mu g.kg^{-1}$ body weight in mice (WHO, 1998). The two variable amino acids in MCLR are leucine (L) and arginine (R) in positions 2 and 4 respectively (Figure 1). These amino acids provide hydrophilic properties to the molecule which is extremely stable at high temperatures and resistant to hydrolysis (Oberholster et al., 2004). This last property enables the toxin to cross through the peptidases in the stomach and the passage of significative amounts of MCLR to the blood stream in an oral intoxication scenario (Chorus and Bartram, 1999). Due to MCLR large size (1000 dalton) and hydrophilic nature, organic anion transporting polypeptides (OATPs) are

required for active uptake of MCLR into the cells (Fisher et al., 2005; Feurstein et al., 2009). Distribution studies of MCLR injected ip in mice revealed a major accumulation of this toxin in the liver (50-70%) followed by the kidneys and intestine responsible for MCLR excretion (Rao et al., 2005). These are considered as the major targets of MCLR toxicity (Wang et al., 2008).

3.4.2. Regulatory aspects

A reference value of 1 µg.L^{-1} of MCLR in drinking water was proposed by WHO in 1998, calculated from the 0.04 µg/kg of body weight TDI value (Tolerable Daily Intake) obtained from sub-chronic studies of oral administration of MCLR in mice (Fawell et al., 1994). This provisional guideline was adopted by many countries as a parametric reference value in their national laws on water. In Portugal this value is included in Decreto-Lei 306/2007 de 27 de Agosto. Although this is a measure that may prevent acute human exposure to MCLR, the implications of low-level chronic exposure to microcystins are not yet known. Nevertheless, the International Agency for Cancer Research classified MCLR as a Group 2 compound (i.e. probably carcinogenic to humans; IARC, 2006).

3.4.3. Carcinogenic potential

Epidemiological studies associated the exposure to microcystins-contaminated water and the occurrence of primary liver cancer (Ueno et al., 1996) or colorectal cancer (Zhou et al., 2002). Besides, evidences of tumour promotion activity induced by MCLR were reported in the liver (Hu et al., 2008), skin (Falconer, 1991) and colon (Humpage et al., 2000) of rodents previously treated with tumour initiators, although the exact mechanism in not known so far. Also, the formation of neoplastic nodules in mouse liver without the previous exposition to an initiator agent has been reported in mice (Ito et al., 1997) suggesting a possible carcinogenic action of MCLR. Furthermore, the ability of MCLR to induce DNA alterations appears to be dependent on the cell type and the toxin exposure concentration, making the genotoxicity properties of MCLR somewhat controversial. DNA damages have been reported *in vivo* in the liver (Rao e 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 (Lankoff et al., 2004; Žegura et al, 2008). However, DNA adducts formation, considered as an indicator of pre-mutagenic lesion, was not induced by MCLR exposure in rat hepatocytes (Bouaïcha et al., 2005). This suggests the existence of an indirect genotoxic mechanism such as the MCLR-induced oxidative stress, rather than direct genotoxic action (Žegura et al., 2003, 2004, 2006 2008; Nong et al., 2007). Additionally, Lankoff et al. (2004) suggested that DNA fragmentation could be a consequence of apoptosis rather than a genotoxic effect.

3.4.4. Cellular targets and mechanisms of toxicity

Microcystins hepatotoxicity is mediated through the inhibition of protein phosphatases 1 and 2A (Honkanen et al., 1990; Mackintosh et al., 1990). These protein phosphatases are responsible for the phosphorylation of key proteins and their inhibition can lead to the hyperphosphorylation of cytoskeletal proteins with the consequent hepatocyte deformation, membrane blebbing, cell shrinkage and rounding, chromatin condensation, and organelle redistribution, classical signs of an apoptotic process (Fladmark et al., 1999; Batista et al., 2003; Herfindal and Selheim, 2006). Necrosis may occur as well (Khan et al., 1995; Hitzfield et al, 2000; Trinkle-Mulcahy and Lamond, 2006).

Traditionally, mitochondria are considered as the main intracellular target of MCLR and several reports have considered it as a central executioner of MCLR-induced apoptosis in hepatic cells (Ding et al., 1998, 2000a, 2000b; Ding and Ong, 2003). The events that take place in this mitochondrial apoptotic pathway include the formation of reactive oxygen species (ROS), decrease of the mitochondrial membrane potential (MMP), membrane depolarization and mitochondrial permeability transition (MPT) pore aperture, with the release of Ca^{2+} and citochrome-c to the cytoplasm (Ding et al., 1998, 2001; Ding and Ong, 2003; Žegura et al., 2004; Nong et al., 2007; Weng et al., 2007). Other mechanisms such as caspases and calpain activation (Fladmark et al., 1999), alterations in the expression of pro-apoptotic and anti-apoptotic Bcl-2 family proteins and p53 gene are also involved in MCLR-induced apoptotic pathway (Fu et al., 2005; Weng et al., 2007; Billam et al., 2008). The involvement of oxidative stress in MCLR-mediated toxicity have been demonstrated by the decrease of reduced glutathione (GSH) and also of antioxidant enzymes such as glutathione peroxidase, glutathione reductase, superoxide dismutase and catalase, as well by the increase of lipid peroxidation in response to MCLR exposure (Moreno et al., 2005; Andrinolo et al., 2008). GSH is a major antioxidant that can act as a free radical scavenger or as an important conjugate of MCLR in detoxifying pathways (Ding et al., 2000a). Its depletion is often accompanied by ROS generation and increased cell susceptibility to MCLR-induced cytotoxicity (Ding and Ong, 2003). These effects altogether are relatively well studied in mice liver *in vivo* (Fawell et al., 1994; Yoshida et al., 1997), in primary cultured rat hepatocytes (Khan et al., 1995; Toivola et al., 1997; Ding et al., 2000b; Mankiewicz et al., 2001; Moreno et al., 2005), human hepatocytes (Batista et al., 2003) and fish hepatocytes (Fisher et al., 2000; Malbrouck et al., 2003; Boaru et al., 2006). However, sensitivity differences to MCLR are reported to exist between rat and human primary cell hepatocytes cultures (Batista et al., 2003). Additionally, comparison of the sensitivity of primary and permanent cell lines to MCLR revealed a 100-fold difference in the concentrations of MCLR necessary to obtain similar effects (Khan et al., 1995; Wickstrom et al., 1995; McDermott et al., 1998).

Furthermore, there is a higher sensitivity to MCLR in hepatic permanent cell lines than non-hepatic permanent cell lines (Chong et al., 2000)

This may be due to the fact that non-liver cells and permanent cell lines exhibit a lower expression, or even loss, of OATP during the process of cell culture. In fact, the transfection of OATPs genes into MCLR insensible permanent cell lines induced cytotoxic effects after toxin exposure (Monks et al., 2007).

3.4.5. Effects of MCLR in non-liver cells

Although MCLR is considered primarily as a hepatotoxin, there have been also recognized effects in non-hepatic cells. However, these appear to be less severe than in hepatocytes, as reported in kidney and intestine cells (Ito et al., 2001; Atencio et al., 2008; Wang et al., 2008).

Nephrotoxicity has been suggested to be a consequence of MCLR accumulation (Wang et al., 2008) and elimination (Nobre et al., 1999) in the kidneys. In fact, the kidneys are responsible for the partial excretion of microcystins in the organism (Robinson et al., 1991) and expression of OATPs was demonstrated in kidney *in vivo* (Hagenbuch et al., 2003). The nephrotoxic activity of MCLR was characterized by alterations in renal function and antioxidant enzyme activity reported *in vivo* in rodents (Nobre et al., 1999; Milutinović et al., 2003; Moreno et al., 2005; Andrinolo et al., 2008). Additionally, Milutinović et al (2003) detected renal alterations equivalent to hepatotoxicity damages (cytoskeletal disruption, apoptosis and necrosis) in kidneys of rats treated with low concentrations of MCLR for 8 months.

Enterotoxicity is also a possible effect of MCLR. The entero-hepatic recirculation of MCLR leads to a reintroduction of the toxin into the small intestine after being transported to the liver (Falconer et al., 1992). Following MCLR exposure, intestinal secretion was observed in rats (Nobre et al., 2004) and enterocyte injury in chickens (Falconer et al., 1992). Additionally, enterocyte apoptosis is reported as well as the presence of MCLR in the villi of mice treated with 75% LD_{50} MCLR for 24h (Botha et al., 2004b). These observations may account for the gastroenteritis symptoms observed in human intoxications by MCLR and confirm that the intestine is indeed a target of MCLR.

The mechanisms mentioned above (section 3.4.4) for liver cells were also described in the kidney *in vivo* (Moreno et al., 2005), in kidney mitochondria isolates (La-Sallete et al., 2008), human lymphocytes (Mankiewicz et al., 2001; Lankoff et al., 2004) and some permanent cell lines (McDermott et al., 1998; Lankoff et al., 2003; Žegura et al., 2008).

In a previous study with the monkey kidney Vero-E6 cell line it was suggested that the ER could be a primary target of MCLR, and that it seemed to be involved in the triggering of autophagy at subcytotoxic MCLR concentrations (Alverca et al., 2009). The same authors

also suggested that this was a cell survival mechanism adopted to eliminate the toxin or/and the MCLR-induced cellular damages. Additionally, lysosomal injuries were shown to occur previously to mitochondrial impairment, contrary to what is the most frequently reported in the literature. In this context, and to better understand the underlying mechanisms of MCLR toxicity, it is of extreme importance to establish if the toxin effects and cellular response mechanisms reported, are transversal to cells from other MCLR-target organs, or on the contrary, are specific to this cell line.

4. Objectives

The cellular mechanisms by which MCLR induces its effects are still not completely understood, particularly in non-liver cells. This study follows the previous work performed in the Vero cell line and tests the hypothesis of an organelle cross-talk responsible for the effects of MCLR, involving the ER, lysosomes and autophagy. For that purpose, *in vitro* models of the main target organs of toxin accumulation/elimination were chosen for comparison of toxic responses: HepG2 (human liver), Vero-E6 (monkey kidney), MDCK (dog kidney) and CaCo2 (human intestine).

The primary aims of this work are:

1) Compare the sensitivity of the permanent cell lines HepG2 (liver), Vero-E6 and MDCK (kidney) and CaCo2 (intestine) to MCLR.
2) Evaluate cytotoxic and morphological MCLR-induced damages and establish a dose/response relation.
3) Detect intracellular targets of MCLR through fluorescence and transmission electronic microscopy (TEM) observation of lysosomes, mitochondria, nucleus, plasma membrane, endoplasmic reticulum and cytoskeleton.
4) Compare of the cellular response mechanisms to MCLR in the different cell types.

MATERIALS AND METHODS

1. MCLR: sources and preparation of stock solutions

Two sources of MCLR were used in this work: semi-purified extracts from a MCLR-producer strain of *Microcystis aeruginosa* (Kützing) and commercial MCLR (named hereafter as pure MCLR). A study of MCLR effects at the concentrations tested in the present work implies the use of large amounts of toxin with a very high cost. For this reason, MCLR semi-purified extract obtained from a *M. aeruginosa* strain was used in all experiments. In parallel, cytotoxity assays with pure MCLR and an extract from a non-toxic *M. aeruginosa* strain were used as positive and negative controls, respectively.

1.1. Semi-purified extracts from *M. aeruginosa*

Strains of *Microcystis aeruginosa* used in this study were isolated in 2000 from Montargil reservoir (Portugal) and have since then been successfully maintained in laboratory conditions (Valério et al., 2009a), within the "Estela Sousa e Silva Algal Culture Collection" of the Laboratory of Biology and Ecotoxicology, National Health Institute Dr. Ricardo Jorge. MCLR was purified from extracts of large-scale cultures of a strain of *Microcystis aeruginosa* (LMECYA 110) characterized as a producer of the MCLR variant of microcystins (Valério et al., 2009b). A non-toxic *M. aeruginosa* strain (LMECYA 127) was also used with biomass[1] concentration equivalent to the biomass concentration of LMECYA 110 strain. This non-toxic LMECYA 127 extract was prepared with the same protocol used for LMECYA 110 extract and provided a negative control. Cultures of LMECYA 110 and LMECYA 127 were grown in plankton light reactors (Aqua-Medic, Bissendorf, Germany) with 2.5L of Z8 medium (Skulberg and Skulberg, 1990) under continuous aeration, at a light intensity of 40 $\mu E.m^{-2}.s^{-1}$ and in a 14h/10h L/D cycle 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 75% methanol (10 mL/100 mg dry weight) overnight at 4°C under magnetic stirring. The extracts were 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 extracts were cleaned-up by solid phase extraction on Sep-Pak C18 cartridges (500 mg Waters, Massachusetts, USA) previously conditioned with 20 mL of ethanol and equilibrated with 20 mL of distilled water. The microcystin-LR containing fraction (and the correspondent fraction of the LMECYA 127 extract) was eluted with methanol at 80% (v/v) and evaporated to dryness. The solid residue was ressuspended in 25 mM acetic acid and manually injected into a Bio-Gel P2 (40–90 mm, Bio-Rad Inc., CA,

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[1] Biomass is considered as the total mass of cyanobacterial cells lyophilized present in the extract and is expressed as mg of dry weight per mL.

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 Well-Chrom 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. The concentration of MCLR on the extracts was determined by fitting the area of the corresponding HPLC-DAD peak to the MCLR calibration curve. The later was constructed by analyzing commercially available MCLR standards (Alexis Biochemicals, CA, SA). MCLR-containing fractions (and the correspondent LMECYA 127 fractions) were evaporated to dryness and ressuspended in bi-distilled water. The final extracts were sterilized by filtration on 0.22 µm syringe filters and kept at -20°C until use. The concentration of MCLR on the final extracts was reanalyzed by HPLC-DAD to contemplate eventual toxin losses during ressuspension and filtration procedures. Extract-work solutions were prepared by serial dilutions of stock extracts in fresh cell line culture medium.

1.2. Pure commercial 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 pure MCLR (1 mM) was prepared by dissolving the toxin in growth medium without Fetal Bovine Serum (FBS) and kept at -20 ^{o}C until use.

2. Mammalian cell lines and culture conditions

2.1. Cell lines maintenance

In this work four mammalian cell lines were used derived from liver, kidney and intestine. The human hepatoma HepG2 cell line (DSMZ ACC 180) has an epithelial morphology and was grown in Dulbecco´s Modified Eagle Medium (DMEM) supplemented with 10% FBS. The Vero-E6 cell line, is a clone of kidney epithelial cells from the African green monkey *Cercopithecus aethiops* (ATCC-CRL 1586) that has fibroblast morphology and was maintained in Modified Eagle Medium (MEM) supplemented with 10% FBS, 1 mM sodium pyruvate and 0.1mM non-essencial aminoacids. The MDCK cell line (ATCC CCL-34) was isolated from a cocker spaniel dog kidney, has an epithelial morphology and was maintained in MEM supplemented with 10% FBS, 1mM sodium pyruvate and 0.1 mM non-essencial aminoacids. The CaCo2 permanent cell line was established from a human intestine carcinoma (ATCC HTB-37). It has an epithelial morphology and it was maintained in RPMI 1640 medium supplemented with 10% FBS.

These cell lines are anchorage-dependent and their growth is inhibited by cell contact. Cell propagation was performed twice a week to dilute the cells into new cell culture

flasks. Cell monolayers with a degree of about 70% confluence were trypsinized (0.05% trypsin-EDTA) for a few minutes and ressuspended in fresh growth medium pre-warmed to 37°C to prevent temperature shock. A small aliquot of the cellular suspension was transferred to new flasks (Orange Scientific, Belgium) containing fresh growth medium at 37°C. In CaCo2 cells, growth medium was replaced between propagations. All cell lines were maintained in a 5% CO_2 humidified incubator at 37°C.

Frozen stocks of the four cell types were maintained throughout this study. For this purpose, cells in exponential growth phase, were trypsinized and ressuspended in fresh growth medium at 37°C. After centrifugation, the supernatant was discarded and the cellular pellet ressuspended in 1-1,5 mL of freezing medium (FBS + 10% DMSO) and transferred to criotubes (Nunc, Roskilde, Denmark). These were maintained at -80°C. All media and supplements were purchased from Invitrogen (Paisley, UK).

2.2.   Cell inoculation and exposure to MCLR

The effects of MCLR on cell viability and cellular ultrastructure were evaluated in all four cell lines. Due to time constrains, and according to the sensitivity of each cell line to the toxin (see section 3.1.) all the remaining procedures were conducted only in HepG2 and Vero cell lines. Cells in exponential growth phase were trypsinized and counted in a haemocytometer by the trypan blue exclusion method (Philips, 1973). HepG2, Vero, MDCK and Caco2 cells were seeded in 96 well plates (Sarstedt, Newton, USA) for the cytotoxicity assay ($1x10^4$ cells per well) and in 6 well plates (Nunc, Roskilde, Denmark) for electron microscopy analysis ($1x10^5$ cells per well). For fluorescence microscopy studies HepG2 and Vero cells ($3.5x10^5$) were seeded in 4 well plates (Nunc, Roskilde, Denmark) over sterile coverslips (Marienfeld, Lauda-Königshofen, Germany). For Western Blot analysis HepG2 ($2x10^6$) and Vero ($1x10^6$) cells were inoculated in 6-well plates.

After 24h incubation for cell adherence and growth, the growth medium was removed and serial dilutions of pure MCLR and MCLR extract in fresh medium containing 1.6 to 100 µM of MCLR were added to each cell line culture for cytotoxicity tests. TEM analysis was performed with MCLR extract in the concentrations of 6 to 100 µM of MCLR. In parallel, similar biomass dilutions of LMECYA127 extract (0.25 to 16 mg dw.ml^{-1}) were also tested by both methods to exclude any cyanobacterial matrix effects. For the fluorescence microscopy and Western Blot experiences, MCLR extract concentrations used were 6, 12, 25 and 50 µM. For each assay, the negative control consisted of cells growing in the corresponding growth medium.

Table 3. summarizes the exposure conditions used in each experimental assay.

Table 3 - Outline of experimental setting conditions used in this study.

Assay	Toxin source	MCLR (µM)	*M. aeruginosa* biomass (mg dw.mL)	Inoculum (cells/well)	Cell line	Culture system
Cytotoxicity	Pure MCLR LMECYA110 LMECYA127*	1; 3; 6; 12; 25; 50; 100 1; 3; 6; 12; 25; 50; 100 -	- 0,25; 0,5; 1; 2; 4; 8; 16 0,25; 0,5; 1; 2; 4; 8; 16	1×10^4	HepG2 Vero MDCK CaCo2	96 well plates
TEM	LMECYA110 LMECYA127*	6; 12; 25; 50; 100 -	1; 2; 4; 8; 16 1; 2; 4; 8; 16	1×10^5		6 well plates
Specific stains IF	LMECYA110	6; 12; 25; 50	1; 2; 4; 8	$3,5 \times 10^5$	HepG2 Vero	4 well plates with coverslips
WB	LMECYA110	6; 12; 25; 50	1; 2; 4; 8	2×10^6 1×10^6	HepG2 Vero	6 well plates

*LMECYA127 was used as a negative control of LMECYA110.

3. Evaluation of MCLR effects

3.1. Cytotoxicity assays

The Neutral Red (NR) cell viability assay was performed in order to compare the sensitivity of the different mammalian cell lines to MCLR, to access the respective cytotoxic thresholds and the toxin concentration range used in further experiments, in order to contemplate non-cytotoxic, low and moderately cytotoxic concentrations. The NR test was chosen because it was previously demonstrated as one of the most sensitive viability assays to evaluate the MCLR toxicity in *in vitro* cell cultures (Pichardo et al., 2005; Bouaru et al, 2006; Alverca et al., 2009; Dias et al., 2009). This assay measures the selective intake and retention of the NR dye by the lysossomes of viable cells by opposition to non-viable cells that cannot retain the NR due to lysosomal membrane damage (Borenfreud and Puerner, 1985). The degree of absorbance measured is correlated with cell viability.

The assay was performed according to the protocol that follows (adapted from Borenfreud and Puerner, 1985).

Neutral Red Assay Protocol:

1. Replacement of growth medium by fresh medium (100 µL).
2. Addition of 10 µL of NR solution (at 50 µg.ml^{-1}, Merk, Darmstad, Germany) and incubation for 3h at 37°C.
3. Washing 2x with PBS.
4. Addition of a solution of ethanol:acetic acid:water (50:1:49) to each well to extract the NR from the lysosomes.

5. Shaking for 15´ and absorbance reading at 540nm (Multiscan Ascent spectrophotometer, Labsystems, Helsinki, Finland).

The cell viability assays were performed in triplicate and the results are presented as the mean ± standard deviation (%) of the three replicates relative to the control. An arbitrary threshold of 50% cell viability was considered as an indicator of a marked cytotoxic effect. Values were analyzed with the t-student´s test; $p < 0.05$ was considered as a statistically significant difference.

3.2. Specific Labelings of cellular organelles

3.2.1. Acridine Orange

Acridine Orange (AO) is a metachromatic dye and a week base that, at low concentrations easily enters the lysosomal membrane of live cells, where it accumulates as it becomes protonated. The interaction with the acidic content results in a red coloration visible at the fluorescence microscope under blue light (Lovelace e Cahill, 2007). To assess the toxic effect induced by MCLR in the lysosomes, HepG2 and Vero cells were stained with AO accordingly to the protocol described underneath. HepG2 cells were observed in a confocal microscope (Leica TCS-SPE) while observations of Vero cells were performed under an Olympus BX60 fluorescence microscope (λ=487 nm) coupled with a CCD camera (Olympus DP11).

Acridine Orange staining protocol:
1. Removal of growth medium and washing for 5' with PBS (pH 7.4) pre-warmed at 37ºC.
2. Incubation of coverslips with AO solution (10 µM in growth medium; Invitrogen), 10', in the dark at 37ºC.
3. Rinsing vigorously for 5' with PBS, 37ºC.
4. Mounting between coverslip and slide.

3.2.2. Rhodamine-123

Rhodamine-123 (Rh-123) is commonly used to specifically stain mitochondria of live cells. This fluorescent probe has a cationic nature that interacts with the electronegative potential of the mitochondrial membrane conferring a green coloration to the mitochondria (Johnson et al, 1980). The toxic effects induced in the mitochondria of HepG2 and Vero cells treated with MCLR were assessed by staining with Rh-123 according to the protocol showed bellow. Observation was achieved in a confocal microscope (Leica TCS-SPE).

Rhodamine-123 staining protocol:

1. Removal of the growth medium and washing with fresh growth medium pre-warmed at 37ºC for 5'.
2. Incubation of the coverslips in Rh-123 solution (15 µg/ml in culture medium; Invitrogen), 10', in the dark at 37ºC.
3. Washing vigorously with growth medium at 4ºC.
4. Mounting between coverslip and slide.

3.2.3. Phalloidin

For the visualization of the MCLR-induced cytoskeletal toxic effects, phalloidin was used as a fluorescence probe for actin cytoskeleton in HepG2 and Vero cells. This mycotoxin binds specifically to the junctions between the subunits of F-actin (Cooper, 1987) and visualization is achieved through conjugation with Alexa Fluor 488® (Invitrogen). The actin filaments staining was performed accordingly to the protocol described below and observation was performed in a confocal microscope (Leica TCS-SPE).

Phalloidin staining protocol:

1. Removal of the growth medium and washing for 5' with PBS (pH 7.4) pre-warmed to 37ºC.
2. Fixation with 3.7% PFA in CBS (Cytoskeleton Buffer with 0,32M sucrose, pH 6.1), 15', rt.
3. Washing 3X 5' with PBS (pH 7.4), rt.
4. Permeabilization with 0.5% Triton X-100 in PBS, 3'.
5. Incubation with AbDil solution, 10', rt.
6. Incubation with 25µM phalloidin diluted in AbDil, 1 h, in the dark, rt.
7. Washing for 5' with 0.1% Triton X-100 in PBS.
8. Labeling with DAPI (0.5µg/ml), 5', in the dark, rt.
9. Washing for 5' with PBS, rt.
10. Mounting between coverslip and slide with Vectashield and sealing with nail polish.

3.3. Protein analysis

3.3.1. Analysis of LC3B and GRP94 proteins by immunofluorescence

In this study, it was used an antibody against LC3B, a key protein of autophagy, for the visualization and study of the MCLR-induced autophagy. This protein is implicated in autophagosome formation and can occur in two forms: LC3B-I, which is cytosolic and LC3B-II, associated with the inner and outer autophagosomal membrane (Kabeya et al., 2000).

Both forms are detected with the rabbit polyclonal α-LC3B primary antibody (Novus Biologicals, Littleton, USA). The antibody working solutions used were 1:100 for HepG2 cells and 1:25 for Vero cells.

For the study of the toxic effects of MCLR in the ER, the rabbit polyclonal α-GRP94 antibody (Acris Antibodies GmbH, Germany) was employed. GRP94 is a glucose-regulated stress protein present in abundance in the ER and utilized as an ER marker (Reddy et al., 1999). The antibody dilutions used were 1/50 for HepG2 cells and 1/10 for Vero cells.

The secondary antibody used in all cases was the α-Rabbit Alexa Fluor® 488 (Molecular Probes, Oregon, USA) diluted at 1:150.

The immunofluorescence protocol was adapted from Alverca et al. (2009) and all fluorescence preparations were observed on a confocal microscope (Leica TCS-SPE).

Immunofluorescence protocol:

1) Removal of the growth medium and rinsing the cells with PBS
2) Fixation in freshly prepared 3.7% PFA in PBS: 30´ for LC3B and 60´ for GRP94
3) Permeabilization with 0.2% Triton X-100 in PBS for 10´ with slight agitation
4) Rinsing 3x5´ with PBS/0,05% Tween 20 (PBS/T)
5) Incubation with the primary antibody diluted in PBS/T for 60´ in a humid chamber
6) Washing 3x5´ with PBS/T
7) Addition of secondary antibody diluted in PBS/T and incubation for 60´ in a humid chamber
8) Washing for 5´ with PBS/T, 5´with PBS and 5´ with bdH$_2$O.
9) Staining with 0.5 µg.ml^{-1} DAPI (4′-6-diamidino-2-phenylindole) for 5´
10) Washing for 5´with PBS
11) Mounting on glass slides with Vectashied (Vector Laboratories, Burlingame, CA, USA) and sealing with nail polish.
12) Observation under a confocal microscope (Leica TCS-SPE).

3.3.2. Analysis of GRP94 expression by Western Blot

GRP94 is a 94 kDa protein present abundantly in the ER, presumably an early subcellular MCLR target (Alverca et al., 2009). The analysis of GRP94 expression by Western Blot was performed in HepG2 and Vero cells exposed to the MCLR-containing semi-purified LMECYA 110 extract following a general SDS-Page/Western Blot methodology. Briefly, cells were washed with PBS and lysed in 50 µl of lysis buffer (40%

Sample buffer[2], 60% H_2O, 1% MgCl2 1M and 1% Benzonase enzyme 100x). Lysates were denaturated at 95ºC for 10´ and stored until use at -20ºC. The protein content of lysates was separated by electrophoresis on 10% SDS-PAGE and transferred to a PVDF (polyvinylidene difluoride) membrane (BioRad). The membrane was blocked with 5% dry milk in TBS/T (0,05% Triton X-100 in TBS) (50 mM Tris-Cl, 150 mM NaCl, 7% HCL at 37%, pH 7.6) for one hour and probed with primary rabbit polyclonal α-GRP94 antibody in the dilutions of 1/200 for HepG2 cells (1h, at rt in a humid chamber) and 1/100 for Vero cells (overnight, at 4ºC). The goat-anti-rabbit peroxidase-conjugate secondary antibody (BioRad) was diluted to 1/3000 and incubated for 1h with agitation. Loading control was achieved by probing the membrane with primary monoclonal mouse α-tubulin antibody (Sigma–Aldrich) at the dilution of 1/10000 followed by secondary goat-anti-mouse peroxidase-conjugate antibody (BioRad) incubation at the dilution of 1/3000 with agitation, both for 1h. All antibodies were diluted in block buffer (5% dry milk in TBS/T). For the band detection, the membrane was treated with ECL (Enhanced Chemical Luminescence) reagents and exposed to X-ray film for radiographic detection. Densitometric analysis were performed with the ImageJ software.

3.4. Transmission Electron Microscopy

For the ultrastructural analysis, TEM protocol was adapted from Alverca et al., 2009. Briefly, both cells in suspension and trypsinized adherent cells were centrifuged at 209 xg for 5 min. The cellular pellet was fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer plus 2.5% PFA in 2.5 mM CaCl2 (pH 7.2), for 90 min at room temperature. Cells were then 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 an ethanol graded series and embedded in Spurr resin (EMS, Washington, USA). Ultrathin cuts were made in a ultramicrotome Leica Ultracut R (Viena, Austria) equipped with a diamond knife Diatome MX1698 (Biel, Suica). Sections were contrasted with saturated uranyl acetate and lead citrate and examined under an electron microscope Morgagni 268D (FEI, Eindhoven, Netherlands). Digital images were acquired with a CCD Mega-View (SIS, Münster, Germany).

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[2] Sample buffer (5x) is composed of 200mM Tris-HCL pH 6.8, 25% Glicerol, 11,25% SDS, 500mM DTT at 5%, 0,01% Bromophenol Blue and H_2O.
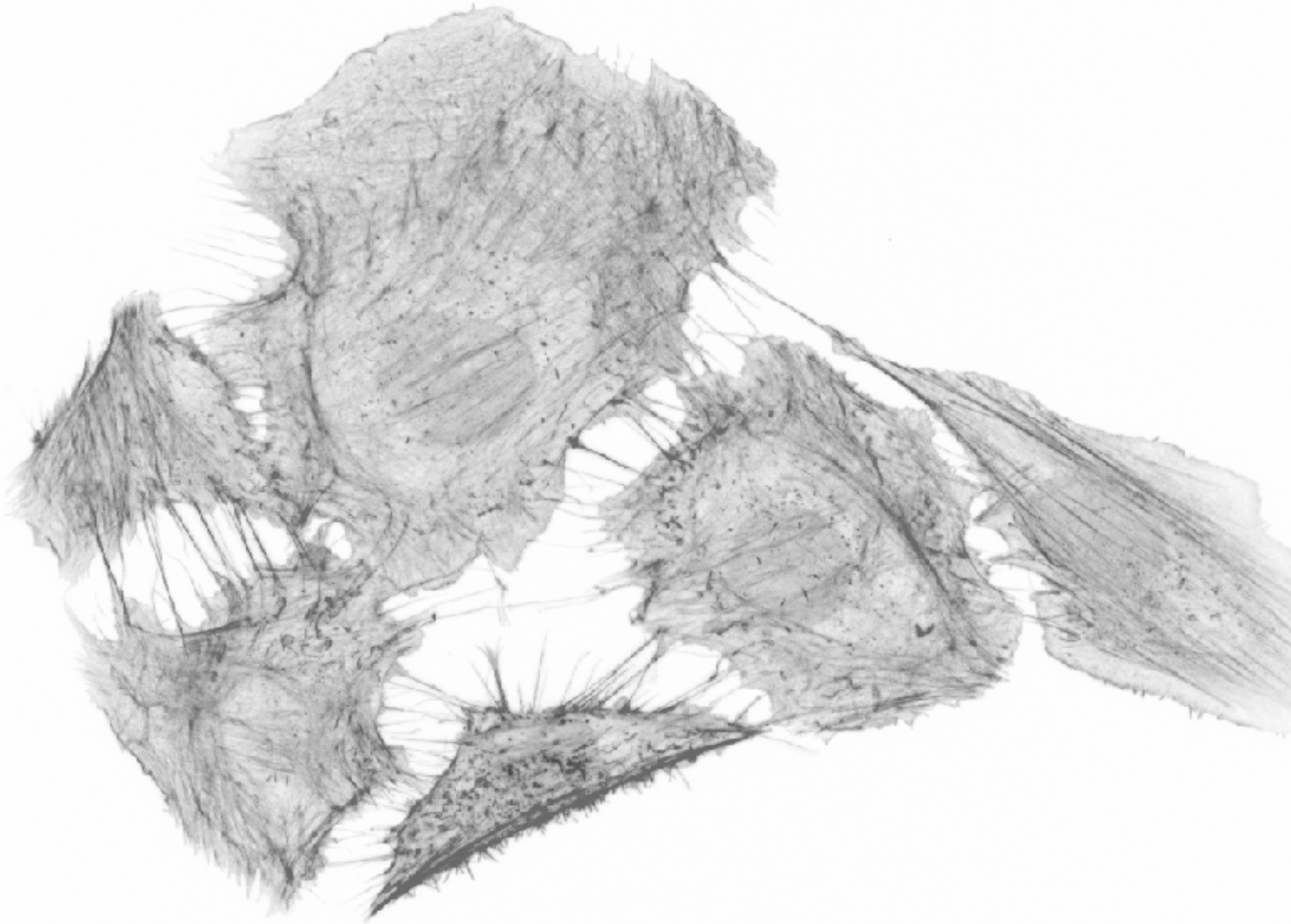
RESULTS

1. Effects of MCLR on cell viability

Cellular viability assays were conducted in HepG2, Vero, MDCK and Caco2 cells exposed to a wide range of MCLR concentrations to establish a cytotoxic threshold and an interval of concentrations to be tested in further experiments with each cell line. All cell lines were treated with LMECYA 110 extract containing 1-100 µM of MCLR. In parallel, cells were also treated with pure MCLR (at the same toxin concentration range) and with the non-toxic LMECYA 127 extract (in the corresponding biomass concentrations of 0,25 -16 mg dw.mL^{-1}) in order to exclude eventual effects from the cyanobacterial matrix and to confirm that the toxicity of LMECYA 110 could be attributed to MCLR.

The effect of LMECYA 110 extract on the viability of HepG2, Vero, MDCK and Caco-2 cell lines is presented in figure 3. For each cell line results are expressed as % of control. For the HepG2 cell line, a significant decrease in cell viability (to 45%) was observed at the concentration of 25 µM MCLR. The cell viability continued to decrease as toxin concentration increased, reaching approximately 20% at 50 µM and 12% at 100 µM MCLR. In Vero cell line a similar concentration-dependent effect was observed but a significant decrease in cell viability occurred only at 50 µM (to approximately 20%). A small reduction in the viability of MDCK and CaCo2 cells was observed at an even higher concentration (100 µM MCLR) and it never decreased below 50% of the negative control.
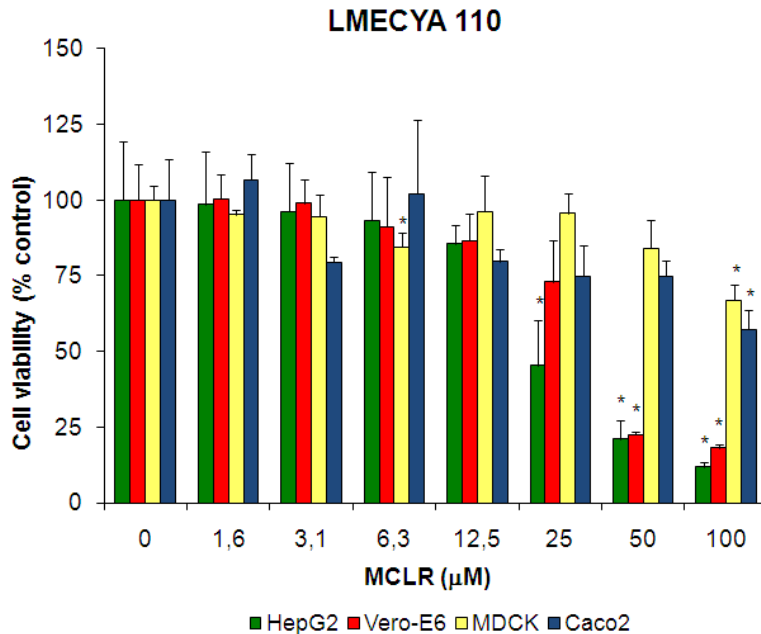
Figure 3 - Viability of HepG2, Vero, MDCK and CaCo2 cells evaluated by the Neutral Red assay, after 24h exposure to the LMECYA 110 extract containing 1.6 - 100 µM of MCLR. Results are shown as mean ± standard deviation of three replicates relative to the control. Asterisk (*) indicates a significant difference from the control p<0.05.

The exposure of each cell line to LMECYA 127 and pure MCLR is shown in figure 4 (A-D). As it can be depicted from this figure, LMECYA 127 did not altered cell viability in any of the studied cell lines. Conversely, pure MCLR induced a concentration-dependent effect comparable to the effect of LMECYA 110 extract, with only minor differences. For HepG2 cells a significant decrease of cell viability induced by pure MCLR was observed above 25 µM (Figure 4A). For Vero cells, a marked decrease in cell viability (≤ 50%) occurred at 50 µM of pure MCLR (Figure 4B). However, the maximum loss of cell viability induced by pure MCLR on HepG2 (53%) and Vero (66%) cell lines was not as pronounced as that induced by LMECYA 110 (88% and 82%, respectively). On the contrary, the effect of pure MCLR on the viability of MDCK cells was more significant as compared with the effect of LMECYA 110 extract, with a slightly decrease in cell viability being induced already at 50 µM and a marked decrease below 50% of cell viability at 100 µM (Figure 4C). The response of CaCo2 cell line to pure MCLR (Figure 4D) was coincident with the response to LMECYA 110 (Figure 3).

In vitro cell culturing is always associated with some variability in the results, as it could be observed in figures 3 and 4. This kind of variability is inherent to the cell cultures procedures (cell counting and inoculation, for example) and is not controlled by the operator. This was particularly experienced with HepG2 and CaCo2 cells.
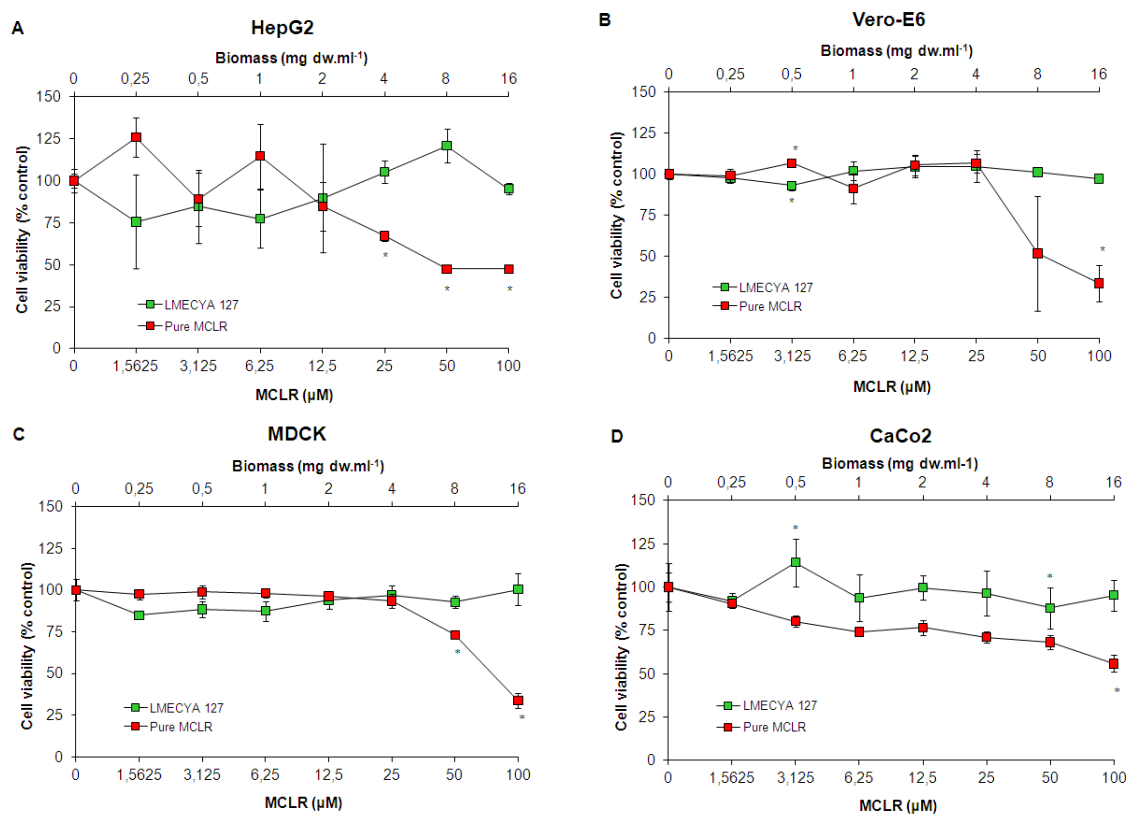

Figure 4 - Viability of HepG2 (A), Vero (B), MDCK (C) and CaCo2 (D) cells evaluated by the Neutral Red assay after 24h exposure to 1.6 - 100 µM of pure MCLR and to the non-toxic LMECYA 127 (the

secondary x-axis represents the corresponding biomass concentrations ranging from 0.25 - 16 mg dw. mL^{-1}). Results are shown as mean ± standard deviation of three replicates relative to the control. Asterisk (*) indicates a significant difference from the control $p < 0.05$.

2. Effects of MCLR on cellular organelles

2.1. Lysosomes

HepG2 and Vero cells exposed to MCLR were labeled with AO in order to study the MCLR- induced morphological alterations in lysosomes.

HepG2 control cells presented numerous uniformly dispersed lysosomes (Figure 5A). After exposure to 6 µM MCLR, cells presented a slight decrease in the number of lysosomes, which in some cases appeared enlarged (Figure 5B, arrows). In cells treated with 12 and 25 µM MCLR, the decrease of lysosomes was more pronounced, whereas more cells presented large red-labeled cytoplasmic lysosomes (Figure 5C and D, arrows). Additionally, at 25 µM MCLR evidence of the lysosomal disruption was detected by the presence of a diffuse cytosolic fluorescence (Figure 5D, asterisk). Lysosomal disruption was almost generalized in HepG2 cells incubated with 50 µM MCLR, which coincided with the decrease both in number and size of labeled lysosomes (Figure 5E).

Untreated Vero cells displayed small and numerous lysosomes, distributed uniformly in the cytoplasm (Figure 5F). A similar labeling pattern was obtained in Vero cells exposed to 6 and 12 µM MCLR, although in the last concentration, the presence of some enlarged lysosomes was also observed (Figure 5G and H, arrow). Following treatment with 25 µM MCLR, the number of lysosomes decreased, but some enlarged lysosomes could still be seen. Additionally some cells showed an increase on the cytoplasmic green labeling, indicative of lysosomal disruption (Figure 5I, asterisk). This effect was more pronounced after incubation with 50 µM MCLR, with most of the cells showing an intense cytosolic fluorescence (Figure 5J).

2.2. Mitochondria

The results from the Rh-123 specific staining of HepG2 and Vero cells are summarized in figure 6. Confocal fluorescence images of HepG2 untreated cells showed rod-like shaped mitochondria with an overall distribution throughout the cytoplasm (Figure 6A). A similar morphology was observed in cells exposed to 6, 12 and 25 µM MCLR (Figures 6B, C and D). Following a treatment with 50 µM MCLR, the mitochondria appeared round, less individualized and the diffusion of Rh-123 to the cytoplasm was evident (Figure 6E). Control Vero cells displayed a perinuclear network of rod-like mitochondria (green-labeled) (Figure 6F). After treatment with 6 and 12 µM MCLR this pattern was maintained, as depicted in

figure 6G and H. Following the exposure to 25 µM MCLR, some cells showed signs of mitochondrial disruption (Figure 6I, arrow) and consequent diffusion of Rh-123 fluorescence to the cytoplasm. This effect was more marked in cells treated with 50 µM MCLR and was accompanied by an alteration of the mitochondrial normal morphology, which appeared rounded (Figure 6J).
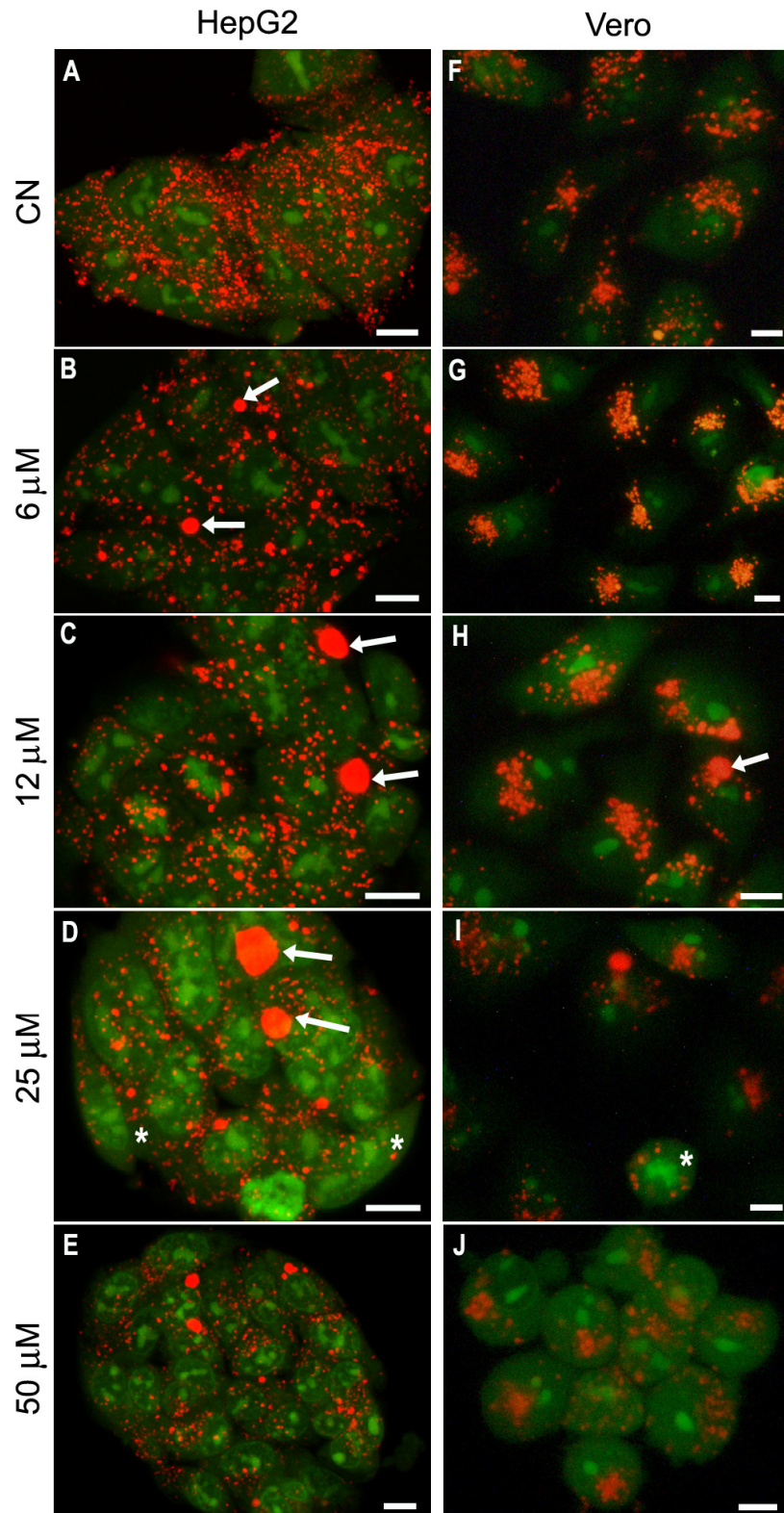
Figure 5 - HepG2 (A-E) and Vero (F-J) cells stained with Acridine Orange after exposure to MCLR displaying red lysosomes. Scale bar represents 10 µm.

HepG2   Vero

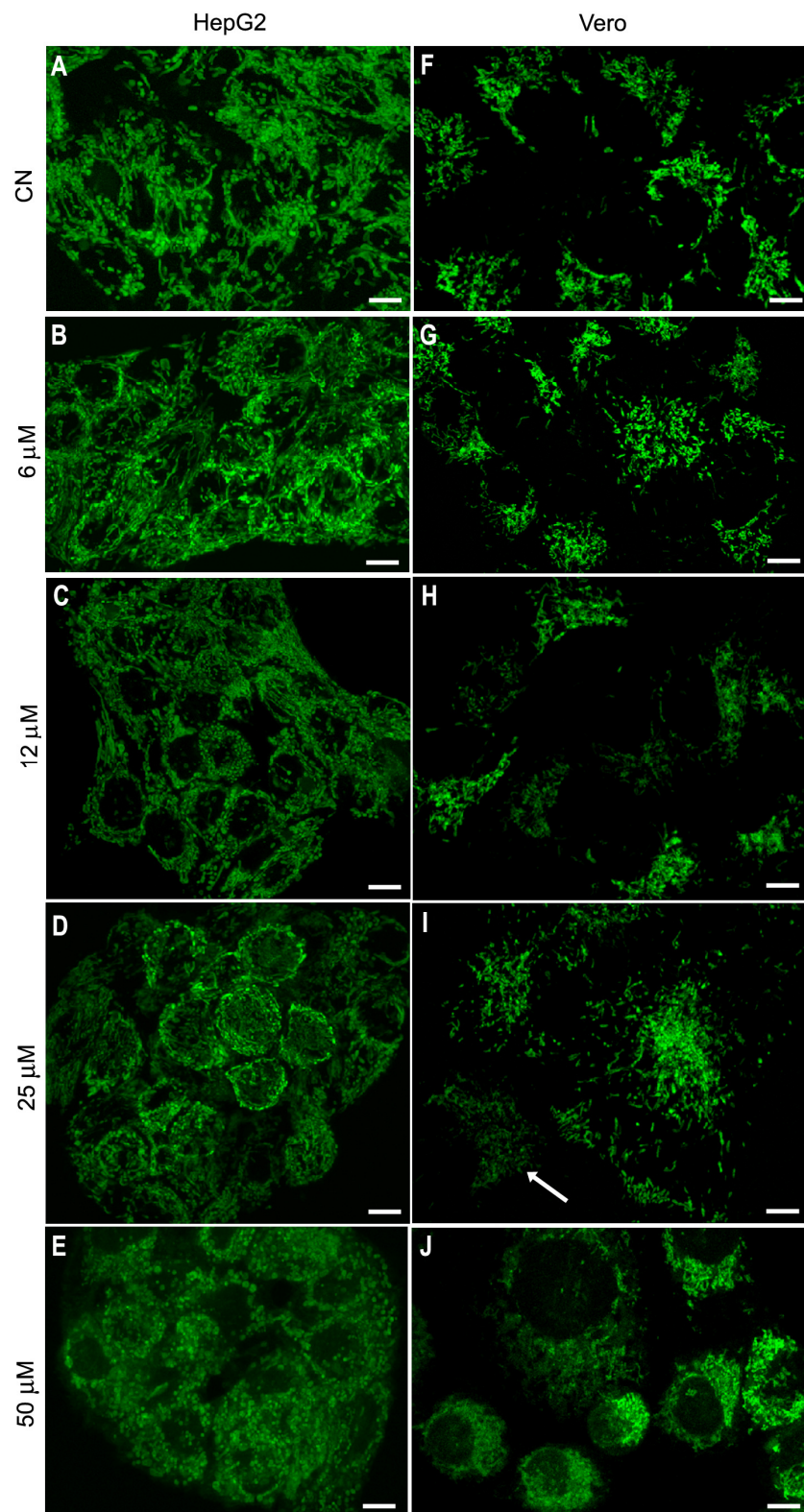**Figure 6 -** Confocal fluorescence images of HepG2 (A-E) and Vero (F-J) cells stained with rhodamine-123 displaying green labeled mitochondria after exposure to MCLR. Scale bar represents 10 μm.
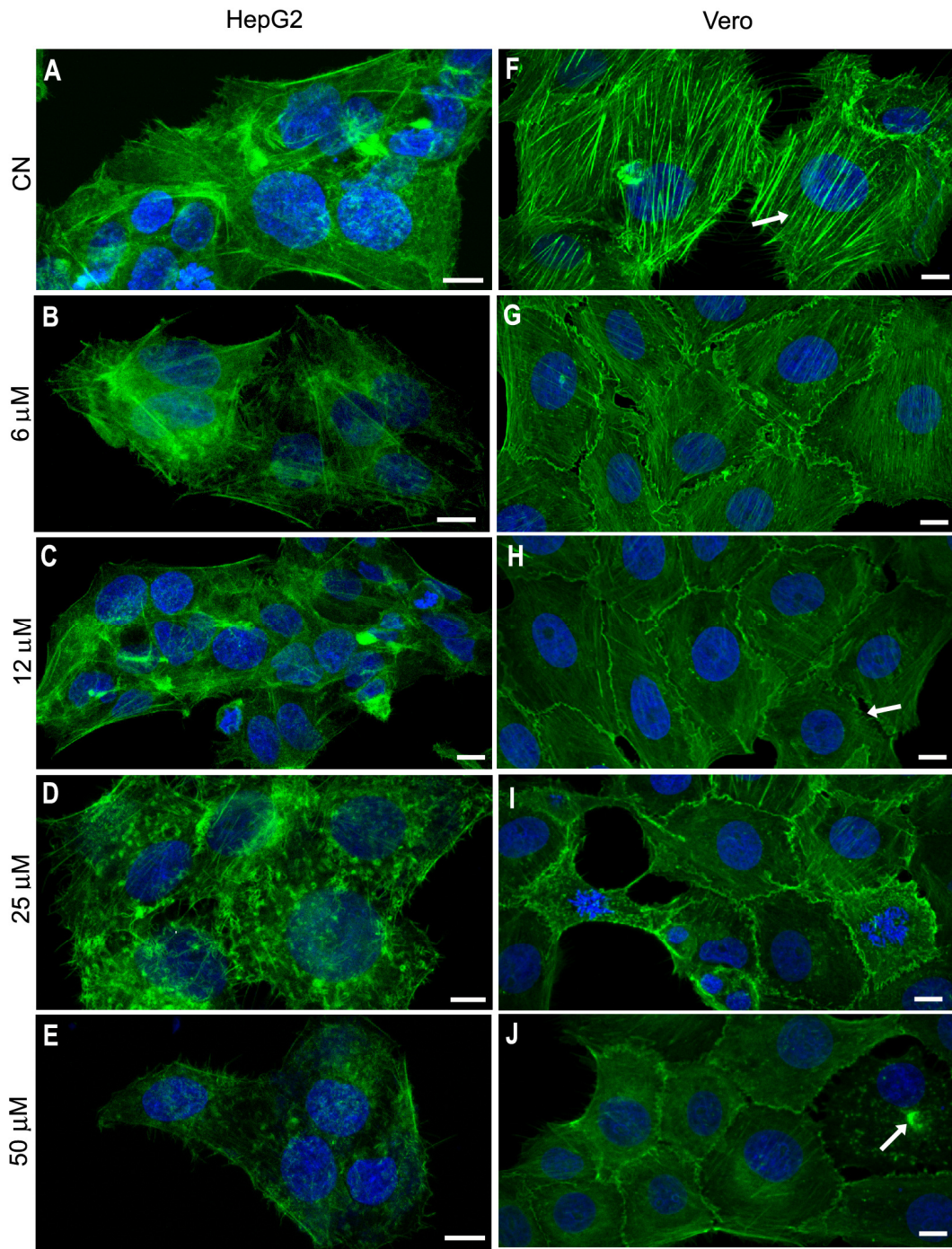
Figure 7 - Actin cytoskeleton of HepG2 (A-E) and Vero (F-J) cells exposed to MCLR for 24h. Scale bar represents 10 µm.

2.3. Microfilaments

Results on phalloidin labeling in HepG2 and Vero cells exposed to MCLR are shown in figure 7. The cells displayed green stained actin filaments and blue stained nuclei.

In non-treated HepG2 cells, actin cytoskeleton had a predominance of a cytosolic actin mesh and peripheral actin bundles. A few stress fibers could be seen across the cells (Figure 7A). A decrease in cytosolic actin filaments and a progressive increase in cortical actin bundles at 6 and 12 μM MCLR was observed (Figure 7B and C). A significant disorganization of the actin filaments could be seen in HepG2 cells exposed to 25 μM MCLR (Figure 7D) with few visible stress fibers. This effect was more marked at 50 μM MCLR (Figure 7E) where the cortical actin fibers were not distinguishable and the overall presence of actin filaments is diminished.

Unexposed Vero cells displayed a cytoplasmic mesh of actin filaments with predominance of stress fibers (intense green filaments, arrow) and dense actin bundles in the cell periphery (Figure 7F). Vero cells exposed to the lowest toxin concentration tested (6 μM MCLR) presented no other changes relatively to the control (Figure 7G). However, at 12 μM MCLR a general depolymerization of microfilaments started to occur with disassembly of peripheral actin bundles (Figure 7H, arrow). These effects were more pronounced following exposure to 25 μM MCLR (Figure 7I). At the highest toxin concentration tested (50 μM MCLR), there was a generalized actin depolymerization, as indicated by the presence of a residual and diffuse cytoplasmic labeling, without the presence of well defined cytoplasmic and peripheral actin bundles (Figure 7J). Additionally, some cells also showed sites of signal accumulation, probably corresponding to the microfilaments collapse (Figure 7J, arrow).

2.4. Autophagosomes (LC3B protein)

Fluorescent labeling with the LC3B antibody marks the two isoforms of this protein: the cytoplasmic form (LC3B-I), observed as a green fluorescence dispersed throughout the cytoplasm and the autophagosomal membrane-associated form (LC3B-II), observed as a green punctuated pattern.

HepG2 control cells displayed a green cytoplasmic-dispersed pattern and a green intense punctuated pattern (Figure 8A). The overlap of fluorescence and differential interference contrast (DIC) images revealed that this punctuated pattern was associated with vacuoles, corresponding to autophagosomes (Figure 8A, arrow). Cells exposed to 6 μM MCLR, showed an increase in the number and size of autophagosomes that appeared enriched in the LC3B-II form, as depicted in figure 8B (arrows). For higher toxin concentrations (12, 25 and 50 μM) although the size of the autophagosomes remained considerably large, the intensity of the punctuated fluorescence labeling reduced

progressively with the increase of MCLR concentration (Figure 8C, D, E, arrows), suggesting a decrease of the autophagic activity.

Control Vero cells exhibited only a green diffuse cytoplasmic pattern (Figure 8F). In Vero cells exposed to 6 µM MCLR, there was the additional punctuated pattern corresponding to autophagosomes (Figure 8G, arrows). The same was observed after incubation with 12 µM MCLR, although a decrease in the accumulation of the LC3B membrane-associated form was evident (Figure 8H, arrow). After exposure to 25 and 50 µM MCLR, most of the cells presented only a cytosolic fluorescence, though less uniform than the control (Figure 8I, J). The overlapped images showed a reduction in the number of autophagosomes.

2.5.   Endoplasmic reticulum (GRP94 protein)

The results of the GRP94 immunolabelling in HepG2 and Vero cells, exposed to the toxigenic LMECYA 110 extract, are presented in figure 9. In control HepG2 cells, diffuse distribution of GRP94 protein is displayed in the cytosol. Additionally, there was a perinuclear accumulation of this protein, observed as an intense green signal (Figure 9A, arrow). HepG2 cells exposed to 6 µM MCLR showed a labeling pattern similar to the control (Figure 9B). However, exposure to higher toxin concentrations (12, 25 and 50 µM MCLR) resulted in a decay on the intensity and number of the perinuclear protein aggregates and a subtle decrease on the cytosolic fluorescence (Figures 9C, D, E). This effect was enhanced with the increase of MCLR concentration.

Vero control cells displayed a widespread and uniform distribution of GRP94 protein within the cytosol, as observed by the diffuse cytoplasmic green labeling (Figure 9F). After treatment with 6 µM MCLR, the labeling pattern was similar to the control cells (Figure 9G). Following exposure to 12 µM MCLR, the GRP94 labeling became less uniform with sites of cytoplasmic signal accumulation, indicating that a redistribution of the protein occurred (Figure 9H). This effect was more marked in cells exposed to 25 µM MCLR and especially to 50 µM MCLR, where the protein accumulation in the perinuclear region was evident (Figures 9I, J, respectively).

HepG2 | Vero
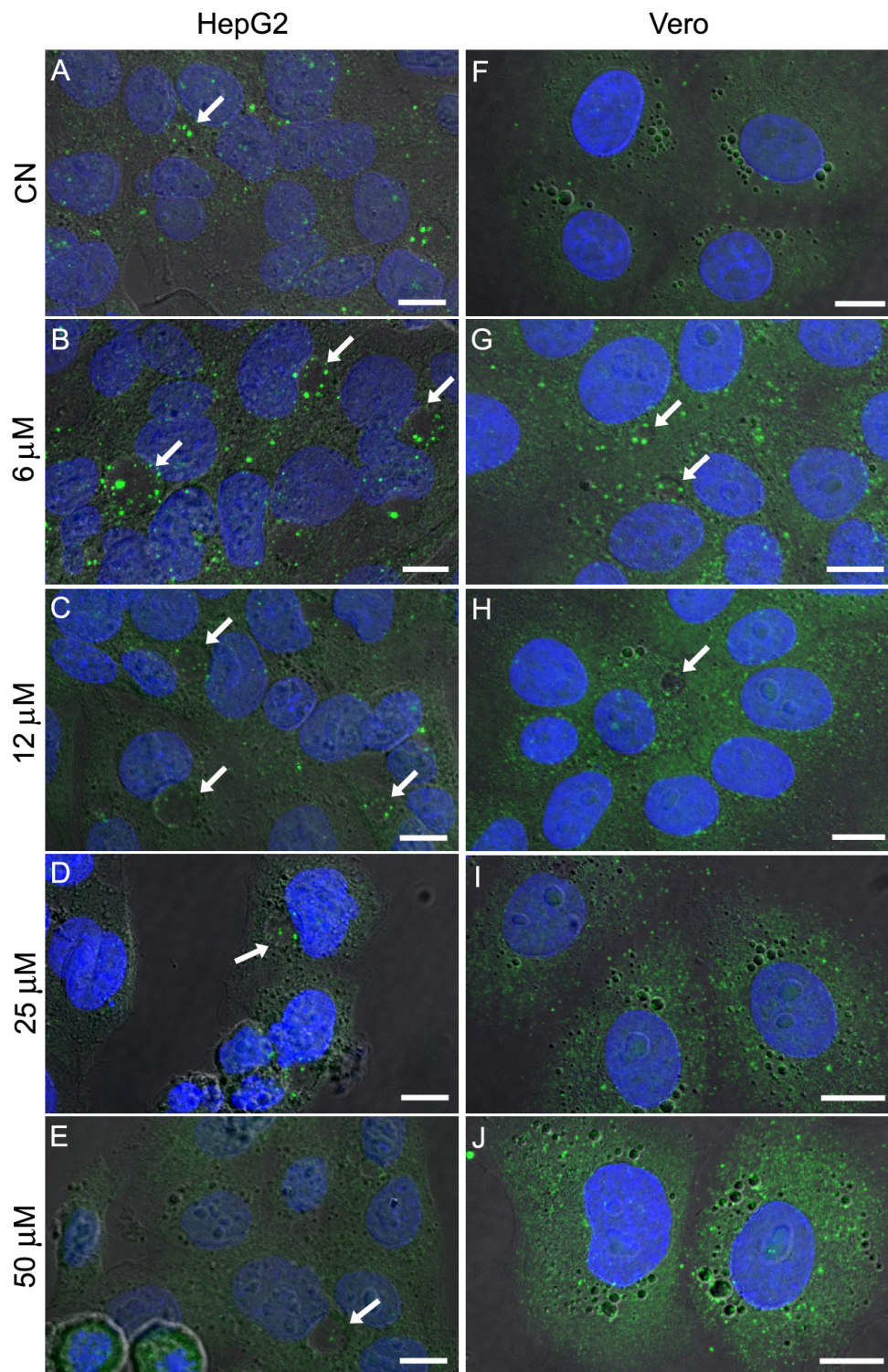
Figure 8 - Immunolabeling of LC3B protein in HepG2 (A-E) and Vero (F-J) cells exposed to MCLR. LC3B protein is labeled green and nuclei are stained blue with DAPI. Arrows indicate autophagossomes localization. Pictures are maximal projections of confocal images. Scale bar represents 10 μm.
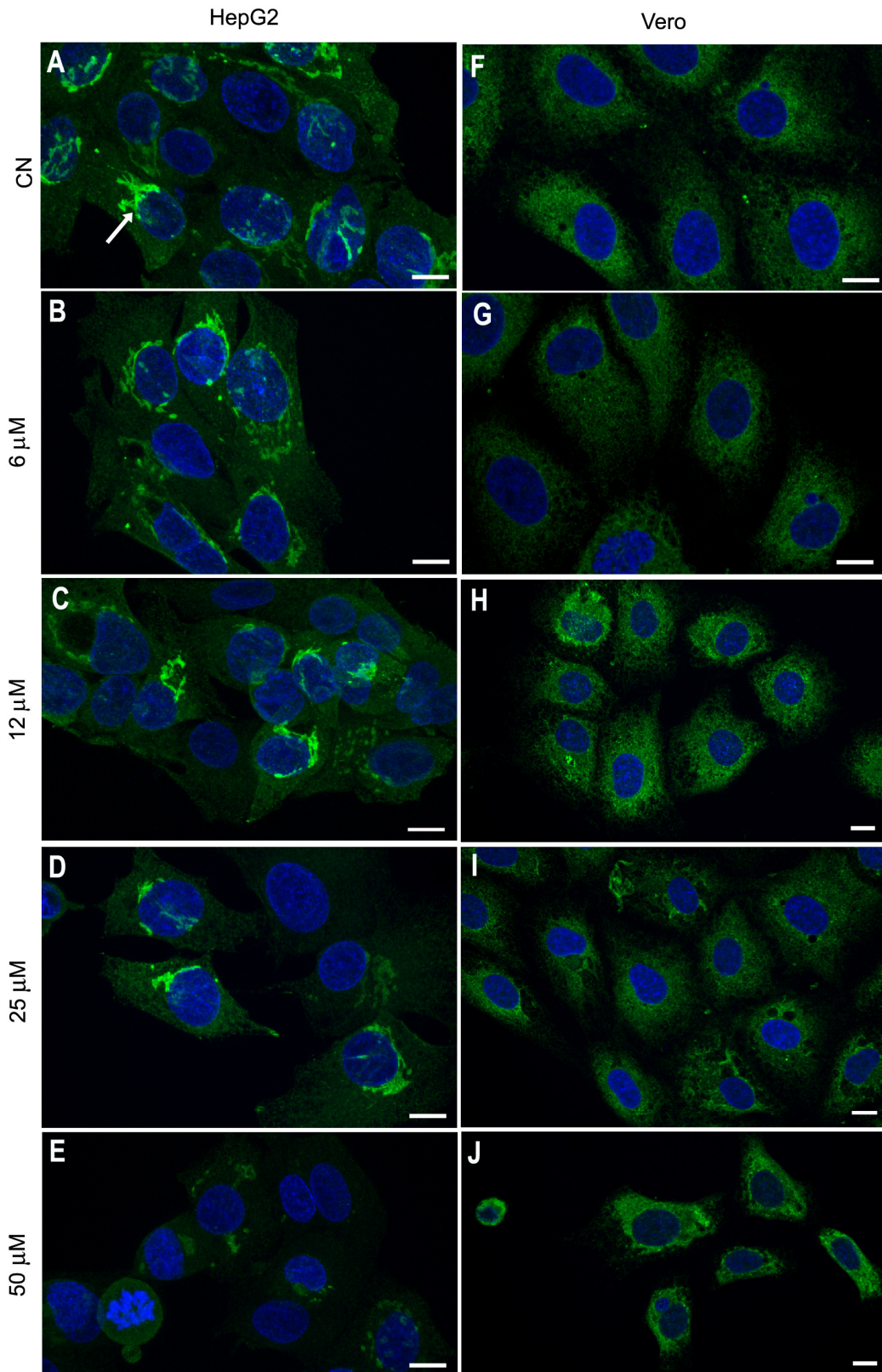
Figure 9 - Immunolocalization images of the GRP94 protein in HepG2 (A-E) and Vero (F-J) cells treated with MCLR. GRP94 is labeled green and nuclei colored with DAPI in blue. Scale bar represents 10 μm.

3. Effects of MCLR on GRP94 expression

Western blot analysis of the expression of GRP94 protein in HepG2 and Vero cells exposed to MCLR is depicted in figure 10. Immunoblot from treated-HepG2 cells shows a MCLR-concentration dependent decrease of GRP94 protein expression (Figure 10A), particularly evident above 12 µM MCLR. Conversely, MCLR did not induce considerable variations on the expression level of GRP94 in Vero cells (Figure 10B). Densitometric analysis of the immunoblots, using α-tubulin as protein loading control (Figure 10C), showed that exposure of HepG2 cells above 12 µM MCLR induced a 20-50% decrease of GRP94 protein expression level. Vero cells treated with MCLR maintained the basal level of GRP94 protein, with no detectable alterations as toxin concentration increased (Figure 10C).

These results were obtained from a single experiment. Thus, data legitimacy should be further validated with, at least, two more independent experiments.
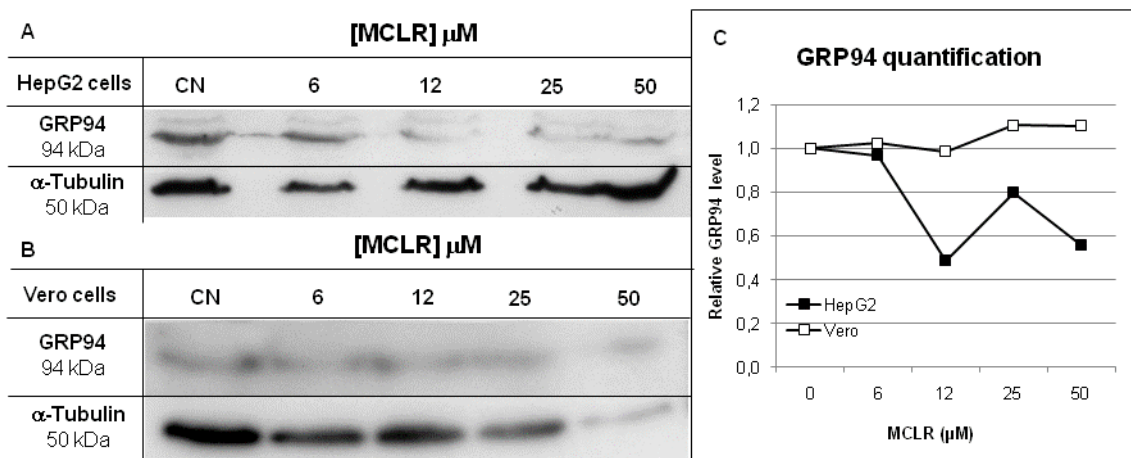
Figure 10 - Expression of the GRP94 protein in HepG2 (A) and Vero cells (B) treated with 6-50 µM MCLR for 24h, evaluated by Western-blot. α-Tubulin was used as loading control. Relative amounts of GRP94 protein in treated HepG2 and Vero cells were quantified by densitometric analysis of the correspondent immunoblots (C). Results were obtained from a single experiment.

4. Effects of MCLR on cellular ultrastructure

The results of the ultrastructural analysis of HepG2, Vero, MDCK and CaCo2 cells exposed to MCLR are resumed in figures 11, 12, 13 and 14 respectively.

The ultrastructural organization of control HepG2 cells is depicted in figure 11A. After exposure to 6 and 12 µM MCLR most of the cells exhibited large vacuoles, occupying a large part of the cytoplasm (Figure 11B, C). Following incubation with 25 µM MCLR some cells displayed vacuolization of the Golgi apparatus (Figure 11D, arrow), while others presented apoptotic features such as the presence of apoptotic bodies (Figure 11E, arrow). Treatment

with 50 µM MCLR induced an increase in the number of apoptotic cells (Figure 11F), as well as mitochondrial damages in still viable cells (Figure 11G). Above this concentration (100 µM), almost all cells presented drastic ultrastructural damages, that in some cases were consistent with an apoptosis death process (Figure 11H) and in other cases typical of necrosis (Figure 11I).

Unexposed Vero cells presented the typical organization, with a large nucleus and cytoplasm with several organelles like mitochondria (M), Golgi apparatus (G) and the endoplasmic reticulum (ER) (Figure 12A). After treatment with 6 µM of MCLR some cells showed ER vacuolization and in some cases its rearrangement, sequestrating a portion of cytoplasm (Figure 12B). Cells exposed to 12 µM MCLR showed numerous cytoplasmic vacuoles, occasionally of large dimensions, with engulfed electro-dense lysosomes (Figure 12C). Exposure to this toxin concentration also induced Golgi's vacuolization, observed in some cells (Figure 12C, insert). At 25 and 50 µM MCLR, mitochondrial ultrastructural alterations, namely cristae vacuolization, were observed (Figsure 12D and E, arrows). The presence of cells with apoptotic features (round and condensed shape, nuclear and cellular fragmentation) became more frequent after treatments with 50 and 100 µM MCLR (Figure 12F, arrow and G).

In MDCK cells, the treatment with 6 µM MCLR did not induce any subcellular damages. In fact, these cells showed the same ultrastructural organization as the control (Figure 13A and B). In some cells exposed to 12 µM MCLR, it was observed a slight vacuolization of the Golgi apparatus (Figure 13C, arrows). This effect was more frequent and evident in cells treated with 25 µM MCLR (Figure 13D, arrows). Additionally, at this toxin concentration mitochondria appeared condensed and in some cases vacuolated (Figure 13D). Treatments with a higher MCLR concentration (50 µM MCLR) induced more severe ultrastructural damages. Some cells presented nuclear fragmentation, highly vacuolated cytoplasm and mitochondria and polarized distribution of the ER (Figure 13E). After exposure to 100 µM MCLR apoptotic cells with apoptotic bodies were more frequently observed (Figure 13F, arrow).

Untreated CaCo2 cells, showed a well organized cytoplasm with several organelles and large glycogen reserves (Figure 14A). No evident cellular damages were observed at 6 and 12 µM MCLR, with treated cells showing the same ultrastructural organization as the control (Figure 14B and C, respectively). The toxin-induced subcellular effects were only observed following the exposure to 25 µM MCLR, and targeted the Golgi apparatus, which appeared clearly vacuolated (Figure14D, arrows). Following the treatment with 50 µM MCLR, some cells also presented condensed and vacuolated mitochondria (Figure14E, arrow). However, it was only after exposure to 100 µM MCLR that the observation of fragmenting, apoptotic cells, became more frequent (Figure 14F, arrow).

Figure 11 - Micrographs of the ultrastructural organization of HepG2 cells treated with different MCLR concentrations. A) Control cells. B) 6 µM MCLR. C) 12 µM MCLR. D, E) 25 µM MCLR. F, G) 50 µM MCLR. H, I) 100 µM MCLR. Scale bar represents 1 µm except in F were it represents 0,5 µm. (N) Nucleus, (M) Mitochondria, (ER) Endoplasmic reticulum, (V) Vacuole, (G) Golgi apparatus.
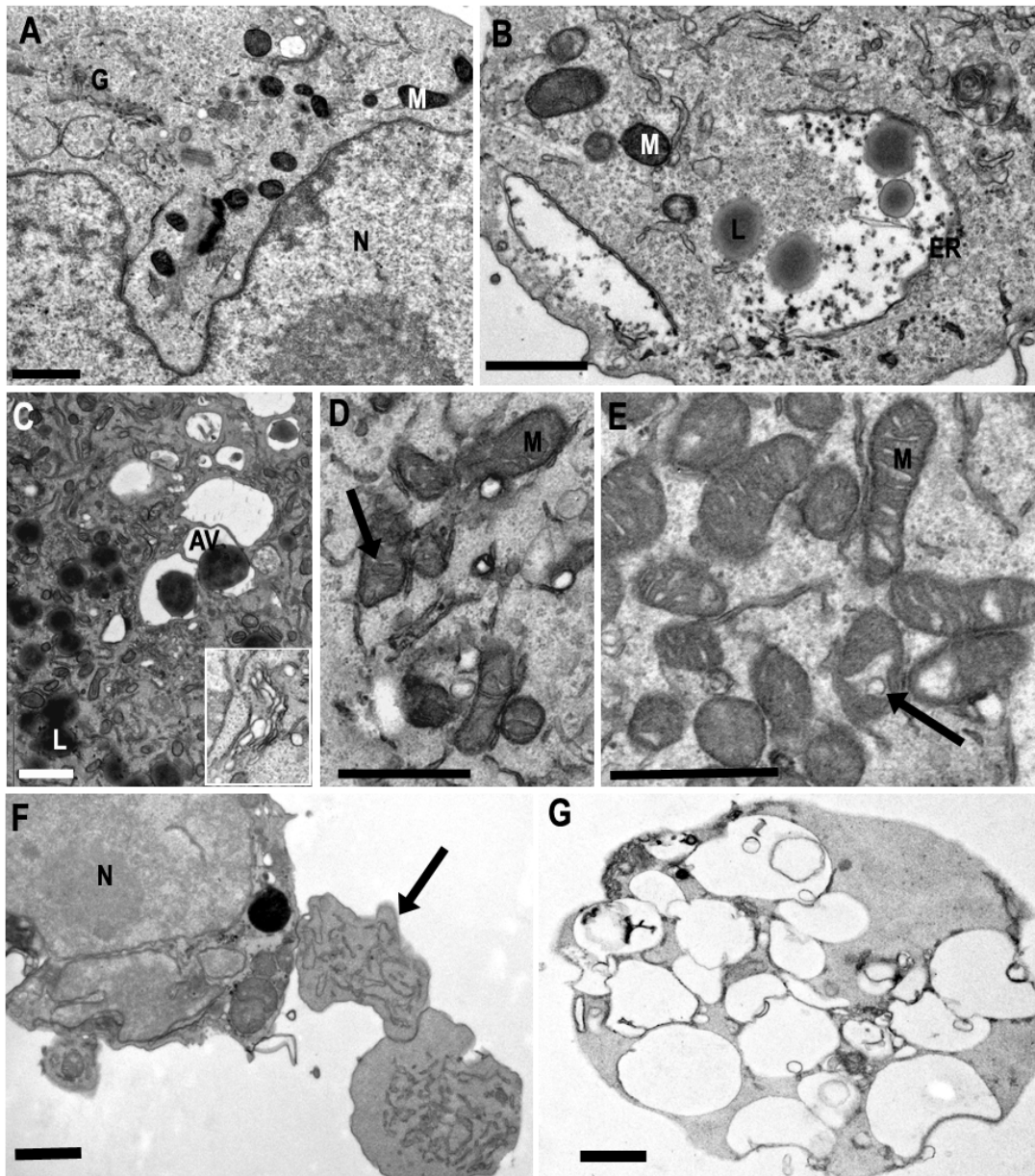
Figure 12 – Micrographs of the ultrastructural organization of Vero cells exposed to increasing concentrations of MCLR. A) Control Vero cells. B) 6 µM MCLR. C) 12 µM MCLR. D) 25 µM MCLR E, F) 50 µM MCLR. G) 100 µM MCLR. Scale bar represents 1 µm. (N) Nucleus, (M) Mitochondria, (ER) Endoplasmic reticulum, (V) Vacuole, (G) Golgi apparatus, (L) Lysosome, (AV) Autophagic vacuole.
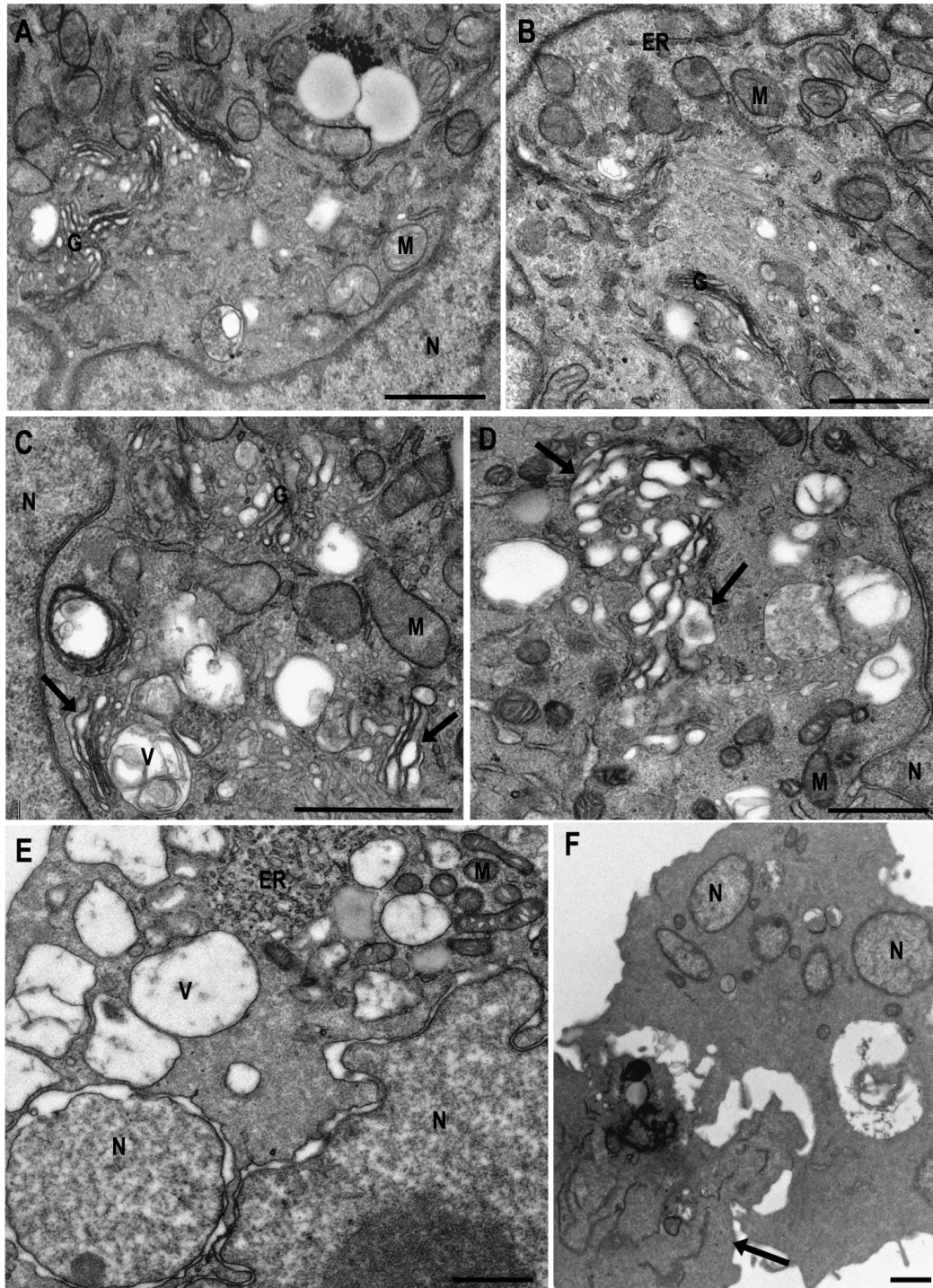
Figure 13 - Micrographs of the ultrastructural organization of MDCK cells after treatment with several MCLR concentrations. A) Control cells. B) 6 µM MCLR. C) 12 µM MCLR. D) 25 µM MCLR. E) 50 µM MCLR. F) 100 µM MCLR. Scale bar represents 1 µm. (N) Nucleus, (M) Mitochondria, (ER) Endoplasmic reticulum, (V) Vacuole, (G) Golgi apparatus.
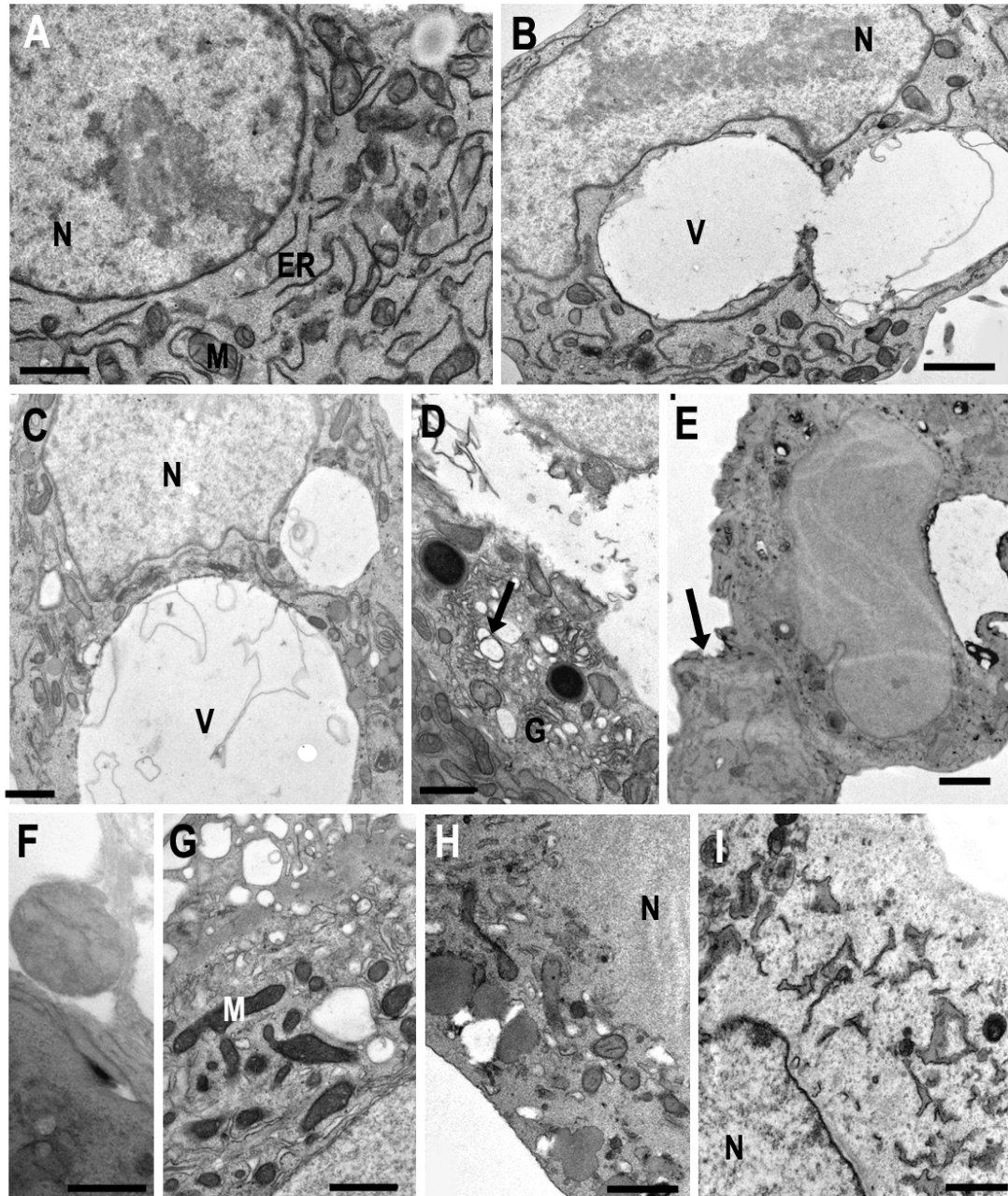
Figure 14 - Micrographs of the ultrastructural organization of CaCo2 cells treated with different MCLR concentrations. A) Untreated cells. B) 6 µM MCLR. C) 12 µM MCLR. D) 25 µM MCLR. E) 50 µM MCLR. F) 100 µM MCLR. Scale bar represents 1 µm. (N) Nucleus, (M) Mitochondria, (ER) Endoplasmic reticulum, (V) Vacuole, (G) Golgi apparatus, (L) Lysosome, (Gl) Glycogen.

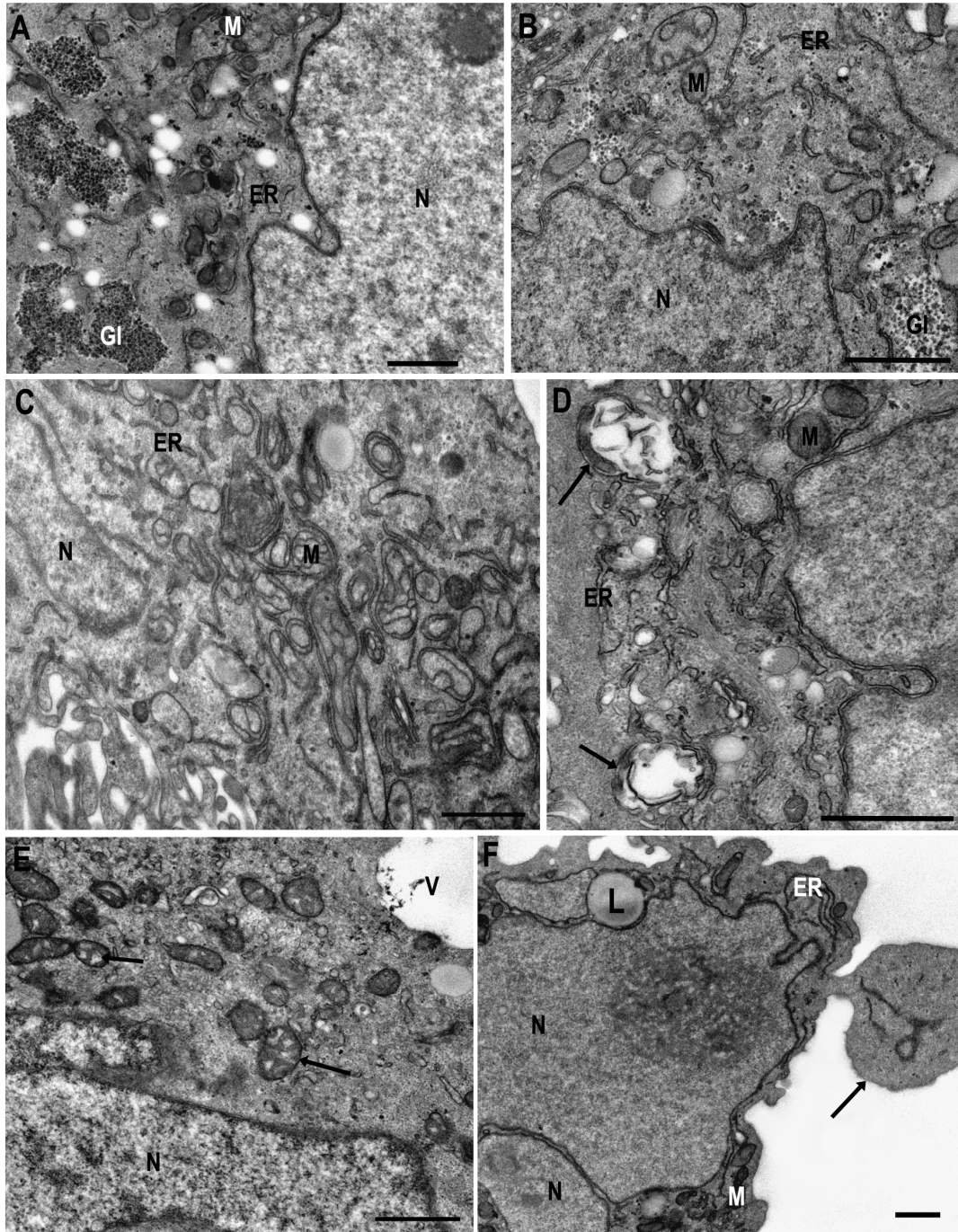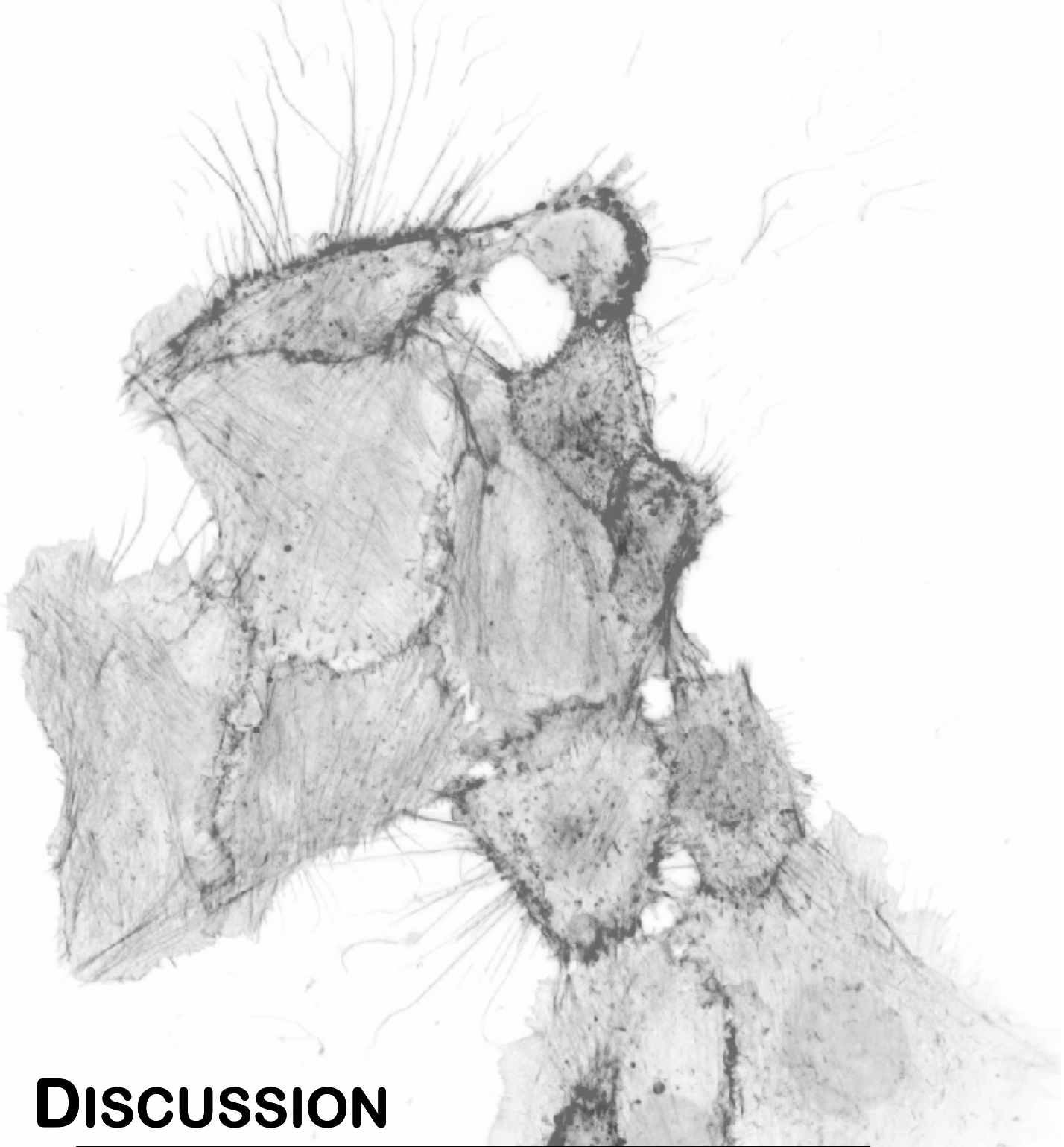# DISCUSSION

In the present study, the effects of MCLR were evaluated in four mammalian cell lines representative of the major target organs of this toxin: HepG2 (human liver), Vero-E6 (monkey kidney), MDCK (dog kidney) and CaCo2 (human intestine).

The study of MCLR toxicity *in vitro* has been somewhat controversial due to, at least in part, the diversity of experimental designs. One of the most important variation factors is the MCLR source. Some studies have been conducted with commercial MCLR whereas the majority of toxicity experiments were performed with extracts from *M. aeruginosa* strains. Although the extracts are widely used for economical reasons (they are less expensive than commercial MCLR), the presence of other putative bioactive compounds has been a matter of some debate (Falconer, 2007). Nevertheless, cyanobacterial extracts may be produced with a high degree of purity through preparative chromatography procedures (Lawton and Edwards, 2001) and the microcystins production by cyanobacterial species can be characterized by analytical (HPLC-DAD, ELISA, LCMS) and molecular tools, such as it was done with the *M. aeruginosa* LMECYA 110 strain used in this study (Valério et al., 2009b).

In the present study, several concentrations of a MCLR extract were tested through the NR viability assay previously reported as a sensible and adequate method to study the effects of MCLR on mammalian (Alverca et al., 2009; Dias et al., 2009) and fish (Boaru et al., 2006) cell lines. The results from the cytotoxicity assays with pure commercial MCLR (95% purity) performed in all four cell types were equivalent to the results of the cytotoxicity assays performed with semi-purified LMECYA 110 MCLR-containing extract. Moreover, exposure to the extract from the LMECYA 127 non-toxin producer did not alter cell viability, excluding the possibility that the viability decays observed in cells exposed to MCLR could be attributed to the bioactivity of a matrix compound present in the extract other than MCLR.

The cytotoxicity results obtained in this study showed that MCLR induced a decrease in cell viability in a concentration dependent manner. The hepatic cell line, as expected, was the most sensitive with a marked cytotoxic response at 25 µM MCLR. It is reported that about 40% of the OATPs found in normal hepatocytes are present in this cell line (Kullak-Ublick et al., 1996; Wilkening et al., 2003). This explains the ability of HepG2 cells to internalize MCLR and justifies its sensitivity to the toxin. However, conflicting results regarding the effects of MCLR in HepG2 cells have been reported, such as the absence of toxicity (Zégura et al., 2003; Bouaru et al., 2006) or toxicity at very distinct dose-ranges (Chong et al, 2000; Nong et al., 2007). This might be explained by the fact that these authors used different biochemical assays such as the MTT and LDH release tests and very distinct incubation conditions (exposition time ranging from 1h to 96h and concentrations of MCLR ranging 0,01 µM to 100 µM MCLR), which difficult the results comparison. Additionally, the variability (metabolic, for example) related to different clones of the same cell line may justify distinct responses

(Knasmüller et al., 2004). Even though, HepG2 cell line can be considered as a suitable model for the liver.

The cytotoxicity results obtained for the Vero monkey kidney-derived cell line showed a significant decrease in cell viability at 50 µM MCLR. This does not agree with previous studies that reported a slightly higher sensitivity of Vero cells compared with HepG2 cells (Dias et al., 2008). This discrepancy may be explained by the fact that, in that study, Dias et al. (2008) used the MTT assay to determine MCLR toxicity, which is based in a different principle than the NR assay. The MTT test reflects the enzymatic mitochondrial activity of viable cells while the NR assay determines the accumulation of the neutral red dye in the lysosomes of viable cells. In fact, in further studies the same authors found a threshold of 50 µM of MCLR on Vero cells, evaluated by the NR assay (Alverca et al., 2009, Dias et al., 2009). Besides cytotoxicity, these authors also found that MCLR induces genotoxicity and organelle damages in Vero cells, recognizing this cell line as an acceptable model for MCLR-induced nephrotoxicity.

In the MDCK dog kidney cell line a pronounced cell viability decay was observed only at the highest concentration tested (100 µM MCLR) while ultrastructural observation revealed mitochondrial damages already at 25 µM MCLR. Again, the nature of the cell viability assay may be questioned and it is probable that if the MTT test had been applied, the cytotoxic threshold for these cells would occur at lower toxin concentrations. In fact, it has already been demonstrated that the different nature of the cytotoxicity assays, conditioned the results obtained (Weyermann et al., 2005; Fotakis and Timbrell, 2006). Besides, the different degrees of toxicity of MCLR observed between the two renal Vero and MDCK cell lines may be the result of inter-species variation (between monkey and dog, in this case) as previously mentioned for other species. For example, mice exhibit a higher sensitivity to MCLR compared to rats (Ito et al., 2001; Wang et al., 2008).

In the literature, MCLR effects in the kidney are restricted to rodents (Nobre et al., 1999; Milutinović et al., 2003; Moreno et al., 2005; Andrinolo et al., 2008; La-Salete et al., 2008) and fish (Kotak et al., 1996; Fisher and Dietrich, 2000). In other mammals there is only one *in vivo* study reporting decreased renal perfusion induced by MCLR in swine (Beasley et al., 2000) and a description of glomerulosclerosis in a dog intoxicated with MCLR (DeVries et al., 1993). Thus, the available data concerning MCLR induced nephrotoxicity is still insufficient leaving much to be investigated in the future.

In the present study, the human intestine CaCo2 cell line also revealed a low degree of sensitivity to MCLR, since a significant cell viability decay was only observed at the highest MCLR concentration tested (100 µM MCLR). This is in agreement with other studies that also reported little concentration dependent effect in CaCo2 cells exposed to MCLR (Chong et al., 2000). However, a significant cell viability decay in these cells was previously

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observed at 10 µM MCLR (Žegura et al., 2008) and 50 µM MCLR (Botha et al., 2004b), although with the MTT assay and higher incubation periods. Nevertheless, the cytotoxicity results from Caco2 cells in the present study were supported by the ultrastructural data, in which the major subcellular damages were only visible at toxin concentrations above 50 µM MCLR. The reports on MCLR intestinal accumulation (Wang et al., 2008), enterocyte apoptosis and increased intestinal water secretion in rodents *in vivo* (Botha et al., 2004a; Nobre et al., 2004) have demonstrated the toxicity of MCLR in the intestine. However, further studies are required to fully characterize the corresponding mechanisms of toxicity.

The overall cytotoxicity results obtained in the present study suggest a decreased order of sensitivity to MCLR from HepG2 to Vero, MDCK and CaCo2 cells. This reflects the organ accumulation pattern of MCLR in mice (Ito et al., 2001) and rats (Wang et al., 2008), in which the liver is the major site of accumulation and the kidney and intestine are secondary targets of the toxin. The different cytotoxic threshold obtained for each cell type tested emphasize that the effects of MCLR are dependent not only on the cellular type but also on the animal species.

The study of the MCLR-targeted organelles has contributed to the understanding of the mechanisms underlying the MCLR-induced citotoxicity. The central executioner in MCLR-mediated cytotoxicity is currently attributed to the mitochondria through oxidative stress induction, involvement of GSH, ROS production, cytochrome-c release and pro and anti-apoptotic proteins of the Bcl-2 family (Ding et al., 2000b; Ding and Ong, 2003; Weng et al., 2007). In HepG2 and Vero cells treated with MCLR, observations of Rh-123 stained mitochondria and TEM revealed that generalized mitochondrial damages occurred only above the cytotoxic threshold in both cell lines (50 µM MCLR). These data indicates that mitochondria, although involved in MCLR-induced toxicity, may not be the main intracellular target of the toxin, as frequently suggested by other authors (Ding et al., 1998; Ding et al., 2000; La-Salete et al., 2008).

Lysosomal damage induced by MCLR is not a much documented effect. However, some studies have already reported an implication of these organelles in MCLR-mediated cellular damage in primary hepatocytes cultures (Boaru et al., 2006; Li et al., 2007). Additionally, a more preponderant role of lysosomes in MCLR toxicity has also been purposed in Vero cells (Alverca et al., 2009). In this study, it was observed that lysosomal morphological alterations started to occur at low toxin concentrations. These alterations were characterized by a reduction on the number of lysosomes and a clear enlargement of some of them, fact that was particularly evident in HepG2 cells. These alterations were coincident with the activation of autophagy (LC3B labeling), also more intense in the hepatic cell line, which indicates that they may result from the fusion of lysosomes into autophagosomes, as supported by TEM images. At higher MCLR concentrations, the results from AO labelling

showed a clear lysosomal disruption that preceded the mitochondrial impairment, in treated HepG2 and Vero cells. This observation, together with the ultrastructural data that evidences the presence of apoptotic cells at the same toxin concentrations, points towards a possible role of lysosomes in the triggering of cell death pathways (Öllinger and Brunk, 1995).

In fact, a primary role of lysosomes in the apoptotic pathway is becoming increasingly more explicit as the number of publications on this subject indicates (Öllinger and Brunk, 1995; Li et al., 2000; Antunes et al., 2001; Zhao et al., 2003). The involvement of lysosomal cysteine proteases, such as cathepsins, and phospholipase A2 have been suggested to interplay in a feedback mechanism that enhances both lysosomal rupture and the production of ROS in the mitochondria (Zhao et al., 2003). In fact, lysosomal membrane permeabilization (LMP) can be initiated by a number of factors such as oxidative stress, microtubules stabilization and increase in the cytosolic levels of calcium (Kroemer and Jäättelä, 2005). Therefore, a lysosomal-initiated apoptosis can induce a mitochondrial-dependent pathway accompanied by citochrome-c release and caspases activation (Ding et al., 2002; Elmore, 2007; Kroemer et al., 2007).

The mechanism of toxic action most associated to MCLR is the inhibition of protein phosphatases 1 and 2A with marked effects in cytoskeletal proteins and cellular architecture (MacKintosh et al., 1990; Batista et al., 2003). In this study, fluorescence labeling of microfilaments revealed a progressive disorganization of the actin cytoskeleton in a MCLR concentration-dependent form in HepG2 and Vero cell lines. The disorganization of the filaments mesh and the depolimerization of the stress fibers in both cell lines was correspondent to the reported effects in hepatic cell lines (Toivola et al., 1997; Batista et al., 2003)

Autophagy is a basal and natural process of protein and organelle turnover (Mizushima et al., 2002; Ziegler and Groscurth, 2004; Kurtz et al., 2007) and is characterized by the sequestration of parts of the cytosol or cytoplasmic organelles into autophagosomes (double-membrane vesicles fused with lysosomes) (Bursch, 2001; Ziegler and Groscurth, 2004; Jahreiss et al., 2008). This cellular process is mainly described as a mechanism of cell survival that enables cells to undergo temporary starvation or to repair inflicted damages (Codogno e Meijer, 2005; Eskelinen, 2005). The ultrastructural characterization of autophagy features has been widely accepted as the hallmark of this cellular process. However, recent studies identified and characterized the microtubule-associated protein light chain 3 (LC3, an essential component of autophagy (Kabeya et al., 2000). Therefore, in this study besides the ultrastructural analysis it was also analysed the immunolocalization of this autophagy protein marker.

In a previous study, an ultrastructural analysis indicated that in Vero cells, exposure to mild MCLR concentrations activated an autophagic cellular response, probably as an

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attempt to eliminate the toxin (Alverca et al., 2009). In this study, these results were confirmed both by TEM analysis and immunolocalization of LC3B protein (Kabeya et al., 2000). Furthermore, it was also demonstrated the induction of autophagy after exposure of HepG2 cells to MCLR. In fact, the presence of autophagosomes was detected in both hepatic and renal cells, but only at subcytotoxic concentrations. Additionally, in HepG2 cells, autophagy seemed to be more intense, as indicated by the presence of very large autophagosomes, highly enriched in the LC3B-II protein. This fact might be related with the strong detoxification functions of hepatocytes, commonly responsible for xenobiotic biotransformation for posterior excretion (Groneberg et al., 2002). Therefore, the role of autophagy as a cell survival strategy upon exposure to MCLR is transversal to HepG2 and Vero cell lines. However, this mechanism does not seem to be effective enough whenever the toxin concentration or the time of exposure is high. At toxin concentrations above 50 µM MCLR, the proportion of cells with apoptotic and, at a lesser extent, necrotic features clearly increased. A functional relationship between autophagy and apoptosis has already been suggested. Depending on the cellular circumstances, autophagy either avoids cell death (suppressing apoptosis) or constitutes an alternative cell-death pathway (Maiuri et al., 2007).

In a recent study, the solution structure of LC3-I was determined and its role as an adaptor protein between microtubules and autophagosomes was suggested (Kouno et al., 2005). This can account for the presence of LC3B-unlabelled cytoplasmic vacuoles at cytotoxic MCLR concentrations. Indeed, this can be the result of the cells` failure to eliminate these vacuoles (Maiuri et al., 2007), due to MCLR-induced microtubule injuries (Toivola et al., 1997).

Despite the advances made in the understanding of the molecular basis of autophagy, the membrane origin of the autophagosomes is still unknown (Luo et al., 2009). Among the several organelles suggested as the source of these membranes is the ER. Our TEM observations showed the enclosing of a cytoplasmic portion by an ER membrane on Vero cells at low MCLR concentrations. Although this data is still not conclusive, it supports the involvement of the ER in the autophagosomes assembly. In the ultrastructural analysis preformed in this study, both MDCK and CaCo2 cell lines showed no signs of autophagy induction or ER alterations at subcytotoxic MCLR concentrations. These data indicate that in these cell lines, the toxin targets primarily other cellular organelles. In fact, at intermediate toxin concentrations (above 25 µM MCLR), MDCK and CaCo2 cells presented mainly Golgi apparatus visible damages. Furthermore, their apparent lack of sensitivity to the toxin, diminishes the MCLR-induced cellular debris (dysfunctional organelles and proteins) making it unnecessary to stimulate a proteolytic pathway, which may justify the absence of autophagy induction in both cell lines. Therefore, it seems that autophagy is not a general but a cell-specific mechanism, involved in the cellular response to MCLR-induced insults.

The endoplasmic reticulum (ER) is primarily recognized as the site of synthesis, folding, and post-translational modification of membrane associated, secreted and some organelle-targeted proteins (Baumann and Walz, 2001). A variety of toxic insults such as oxidative stress, glucose starvation, chemical toxicity, inhibitors of glycosylation, alterations in intracellular Ca^{2+} levels cause ER stress and consequent accumulation of unfolded proteins within the ER lumen (Bando et al., 2005; Dey et al., 2006; Szegezdi et al., 2006). The major protective and compensatory mechanism during ER stress is the unfolded protein response (UPR), a complex cellular pro-survival response that reduces unfolded proteins and restores normal ER functioning (Schroder and Kaufman, 2005). The principal UPR executioners are a set of ER resident calcium-dependent chaperone proteins with anti-apoptotic properties, such as the glucose-regulated protein-78 (GRP 78) and GRP94 (Reddy et al., 1999; Lee, 2001). These proteins are expressed at significant levels in cells under normal growth conditions, but they are specifically up-regulated in response to ER stress (Water et al., 1999; Chen et al., 2002; Bando et al., 2004; Lim et al., 2005; Peyrou and Cribb, 2007).

It is extensively described that oxidative stress plays a central role on MCLR-induced toxicity in mammalian cells (Ding and Ong, 2003). The increase of oxidative stress by MCLR has been associated with GSH depletion. MCLR is metabolized by conjugation with GSH in a reaction catalyzed by glutathuione-S-transferase, which results in the decrease of the GSH intracellular pool and, consequently, in the decrease of cellular antioxidant defense and increase of oxygen reactive species (Pflugmacher et al, 1998; Ding and Ong, 2003). Several studies have characterized the role of the ER, and in particular of the GRP proteins, in the cellular response to oxidative stress (Liu et al., 1997; Ron, 2002; Hung et al., 2003).

Additionally, in a previous work, it was suggested that the ER was the first intracellular target of MCLR-induced toxicity in the monkey renal Vero-E6 cells (Alverca et al, 2009). Therefore, it seemed of interest to study the GRP94 protein distribution and expression under several MCLR exposure conditions in both HepG2 and Vero cells.

According to the results of the present study, MCLR failed to display any up-regulation of GRP94 expression in both HepG2 and Vero cells. On the contrary, in HepG2 cells, data suggests an association between MCLR-induced oxidative stress and suppression, rather than induction, of GRP94 expression. Similar results were reported in HepG2 cells overexpressing CYP2E1 that displayed increased ROS production and lipid peroxidation (Dey et al., 2006) and in primary neuronal cell cultures exposed to H_2O_2 (Paschen et al., 2001). Furthermore, these authors suggested that down-regulation of the UPR and associated GRPs diminished cell tolerance to severe forms of stress, and therefore increased cell commitment to death. In recent years a strong link between the ER and the signalling of apoptosis has been established, and the molecular mechanisms involved in the

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process are now beginning to be unravelled (Szegezdi et al, 2006). In fact, it has been shown that if ER stress is too persistent or too intense that cannot be resolved, the UPR signals change from pro-survival to pro-apoptotic. This has been related with the decreased expression of GRP proteins (Groenendyk and Michalak, 2005; Szegezdi et al., 2006). Furthermore, and besides its association with mitochondria, Bcl-2 proteins have already been reported in the ER membrane, where it appears to function as regulators of reticular Ca^{2+} stores (Thomenius and Distelhorst, 2003). In a recent work with okadaic acid (a phycotoxin that like MCLR strongly inhibits PP1/PP2A), Lin et al. (2006) showed that the toxin-induced inhibition of PP2A resulted in an increase of Bcl-2 protein phosphorylation and its posterior selective degradation by the proteasome. As the maintenance of low concentrations of Ca^{2+} inside the ER is mediated by Bcl-2 proteins, a decrease in its quantity leads to calcium increase in the ER lumen, a pro-apoptotic signal leading to activation of mitochondrial triggered apoptotic pathways such as calpains activation, MPT induction, cytochrome-c release and activation of pro-apoptotic proteins of the Bcl-2 family (Thomenius and Distelhorst, 2003).

As mentioned previously, in the present study, the HepG2 cell line showed to be the most sensitive to MCLR effects when compared with Vero, MDCK and Caco2 cells. This cell line showed a marked decrease of cell viability between moderate to high MCLR concentrations, coinciding with the lower expression of GRP94. Additionally, this decrease of cell viability was related to the ultrastructural appearance of apoptotic cells. Therefore, there is an indication that in HepG2 cells, the induction of apoptosis might be ER-stress mediated. Nevertheless, further studies are necessary in order to clarify this aspect.

In this study, Vero cells displayed a basal level of GRP94 independently of the MCLR concentration, as shown by western blot analysis. This contrasts with the HepG2 cell data, and indicates that the protection efficiency of the cellular ER stress response is cell type specific (Cribb et al, 2005). It has been largely described that the ER is a subcellular target of toxic compounds and that ER stress response plays an important role in xenobiotic-induced nephrotoxicity (Hung et al., 2003; Lorz et al., 2004; Cribb et al., 2005; Peyrou and Cribb, 2007; Kitamura, 2008). Therefore, it was surprising that MCLR did not induce an over-expression of GRP94 in treated Vero cells. In the experimental design performed in this study, the GRP94 expression was only assessed at one time point (24 hours after toxin exposure). Considering that GRPs have been shown to possess a short stable half life and tolerate stress in a limited period of time (Rao et al, 2005), it is then possible that in these experiments the maximum protein expression was not observed. For definitive conclusions, a kinetic analysis should be performed.

The GRP94 protective functions against apoptosis have already been described (Reddy et al., 1999). In light of this, it seems reasonable to assume that in Vero cell line, the

apoptotic signalling in not ER-mediated. In fact, at high MCLR concentrations, when cell viability was markedly decreased and the proportion of apoptotic cells increased, the GRP94 levels remained similar to the control. As demonstrated by the fluorescence and TEM results, at these toxin concentrations (above 25 µM MCLR), the cells presented damages in several organelles, namely in mitochondria, suggesting their involvement in the apoptotic pathway.

A MCLR concentration-dependent redistribution of GRP94 in Vero cells was observed, particularly visible at 50 µM MCLR. This ER rearrangement can be the result of the depolimerization and retraction of cytoskeleton components, resultant from the PP1 and PP2A MCLR-mediated inhibition (Toivola et al., 1997), already reported previously in Vero cells at 40 µM MCLR (Alverca et al., 2009). In fact, a high interdependent association between the cytoskeleton and the ER network has been proved before (Terasaki et al., 1986). This was not seen in HepG2 cells in this study, probably due to the differences in the native GRP94 protein distribution, which in these cells is more heterogeneous with perinuclear sites of accumulation.

The present study of the GRP94 protein expression did not clearly demonstrate that MCLR targets the ER. However, a possible protective role of the ER stress response in MCLR-induced toxicity cannot be discarded in both HepG2 and Vero cells. As previously mentioned, there are other pivotal ER stress proteins, such as GRP78, whose expression profile was not analysed. Additionally, there is accumulating data indicating that ER stress is a potent trigger of autophagy, and that this degradation system is an alternative form to alleviate stress resulting from aberrant and/or misfolded protein accumulation within the ER (Yorimitsu and Klionsky, 2007). In fact, and according to the results obtained in this study, there was an induction of autophagy in both HepG2 and Vero cell lines at MCLR subcytotoxic doses, more evident in the HepG2 cells. These data suggests that in these two cell lines the ER stress response is modulated, at least at low toxin concentrations, via autophagy activation, with the UPR representing a secondary role, if any.

The data here presented confirmed that there is a crosstalk between several organelles such as the ER, mitochondria and lysosomes, in the cellular response to MCLR-induced cytotoxicity. However, this response seemed to be highly dependent on the exposure conditions and on the cellular characteristics. In fact, no pattern of response could be established, even between cells from the same type (Vero and MDCK).

As previously mentioned, the assessment of MCLR effects on the intracellular organelles of MDCK and CaCo2 was not carried out. Therefore, in future work, it would be important to complete this information so that the different cell lines can be fully compared. As well, it would be of interest to further support the study on the endoplasmic reticulum stress protein GRP94, and complement it with the analysis of the GRP78 expression, under several toxin concentrations and times of exposure. The intracellular calcium homeostasis

seems to be relevant in MCLR toxicity. Therefore, the assessment through fluorescence labeling of its dynamic upon exposure to the toxin, could be of interest. Clarification of the Bcl-2 involvement in mitochondria and ER could help understand the organelle crosstalk associated to MCLR.

CONCLUSIONS

The sensitivity of the mammalian cell lines derived from liver, kidney and intestine to MCLR was accessed in this study. All four cell lines presented a MCLR concentration-dependent effect in cell viability, with a decreased order of sensitivity to MCLR from HepG2 to Vero, MDCK and CaCo2 cells. This reflects a similar pattern of MCLR accumulation and toxicity *in vivo*, in which the liver is the major target and the kidney and intestine are secondary organs of toxicity. The suitability of these cell lines as cellular *in vitro* models of target organs of MCLR toxicity was therefore emphasize.

Transmission electronic microscopy analysis showed that MCLR triggers apoptosis in all cell lines. By the contrary, induction of autophagy was only common to HepG2 and Vero cell lines. This demonstrates that autophagy is not a general cellular mechanism against MCLR injury.

Fluorescence and transmission electronic microscopy analysis of HepG2 and Vero cells treated with MCLR enabled to identify several intracellular targets of the toxin. The effect of MCLR on cellular organelles was strongly dependent on the toxin concentration:

1) Autophagy is a defense response to subcytotoxic concentrations of MCLR, particularly in the hepatic cell line;

2) Lysosomes have a dual role on MCLR-mediated toxicity in both HepG2 and Vero cells:

   i. At subcytotoxic MCLR concentrations are involved in autophagic response;
   ii. At higher MCLR concentrations are targeted previously than mitochondria, being possibly early executioners of MCLR-induced apoptosis.

3) The endoplasmic reticulum response was dependent on the cell line:

   i. In HepG2 cells it appears to be involved in the MCLR-induced autophagy or apoptosis processes in a concentration-dependent manner;
   ii. In Vero cells the ER seems to be involved only in the autophagic response.

4) The cytoskeleton is progressively disrupted as concentration increases in both HepG2 and Vero cells.

In summary, several organelles are involved in MCLR toxicity, although their role on cell response to the toxin and the triggered mechanisms seem to be cell-specific.
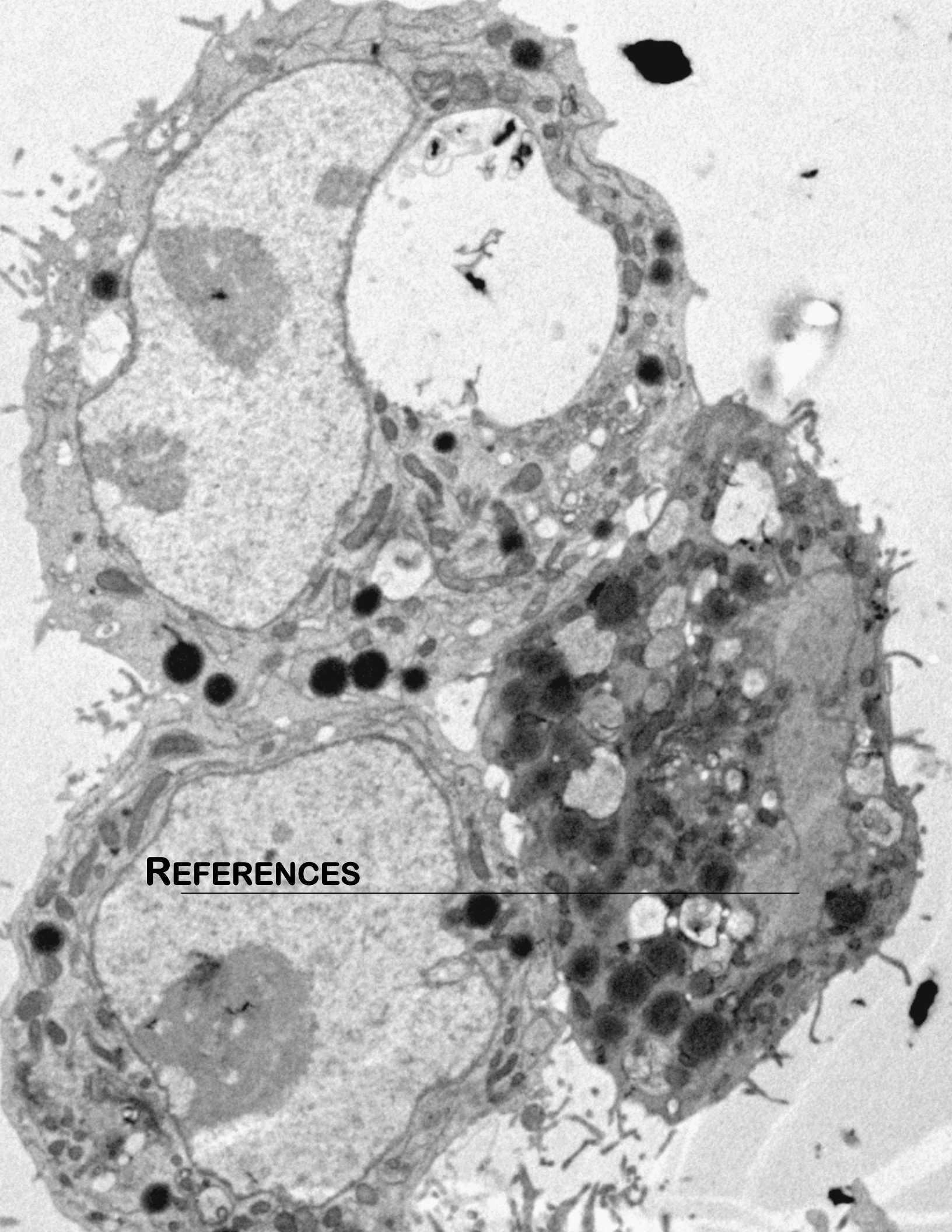
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