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The Kidney Vero-E6 Cell Line: A Suitable Model to Study the Toxicity of Microcystins

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1. Introduction

Microcystins (MCs) are toxins produced by cyanobacteria from water environments that can induce acute and chronic effects on humans and animals, after ingestion/contact with contaminated water [1]. This group of cyclic heptapeptides comprises approximately 80 variants, being microcystin-LR (MCLR) the most frequent and toxic variant [1]. MCs are mainly known for their hepatotoxicity due to their inhibitory activity of serine/threonine phosphatases PP1 and PP2A [2]. This inhibition interferes with hepatocyte homeostasis and structure, leading to the collapse of liver tissue organization, liver necrosis and hemorrhage (Figure 1), which can culminate, in severe cases, in the death of the intoxicated individuals [3, 4].

It has been reported that microcystins cross cell membranes through the transmembrane solute carriers transport family OAPT (Organic Anion Polypeptide Transporters), in particular the OATP1B1, OATP1B2, OATP1B3 and OATP1A2 [7, 8]. They are responsible for the sodium-independent uptake of large amphipathic endogenous and exogenous organic anions into cells and across the blood-brain barrier [9, 10]. The knowledge on the mechanism(s) of OATP-mediated transport is still scarce [9] however, the available information suggests that, in a general way, OATPs act as organic anion exchangers [11], functioning in a rocker-switch type of mechanism and translocating the substrate through a central positively charged pore [9].

Some OATPs are expressed ubiquitously, whereas others are expressed in a tissue-specific way [10]. In fact, the organotropism of MC is due to the selective uptake of microcystins by the OATPs that are primarily expressed in liver [8], such as those mentioned above. For this reason, the study of the toxicological properties of microcystins has been conducted mainly in liver cells *in vivo* and cultured hepatic cells *in vitro*. However, OATP expression in other organs has also been reported, like the case of OATP1A2 in the kidneys [7, 8, 10, 12] and an increasing number of studies have been showing that MCLR can indeed induce nephrotox-

icity [13, 14, 15]. In fact, although MCLR is mostly accumulated in the liver and excreted by biliary route, a fraction of the toxin (9%) is filtrated in the kidneys and eliminated through the urine [16], which makes the kidneys a potential target for MCLR toxicity. In figure 2 is depicted the toxicokinetics of microcystins.
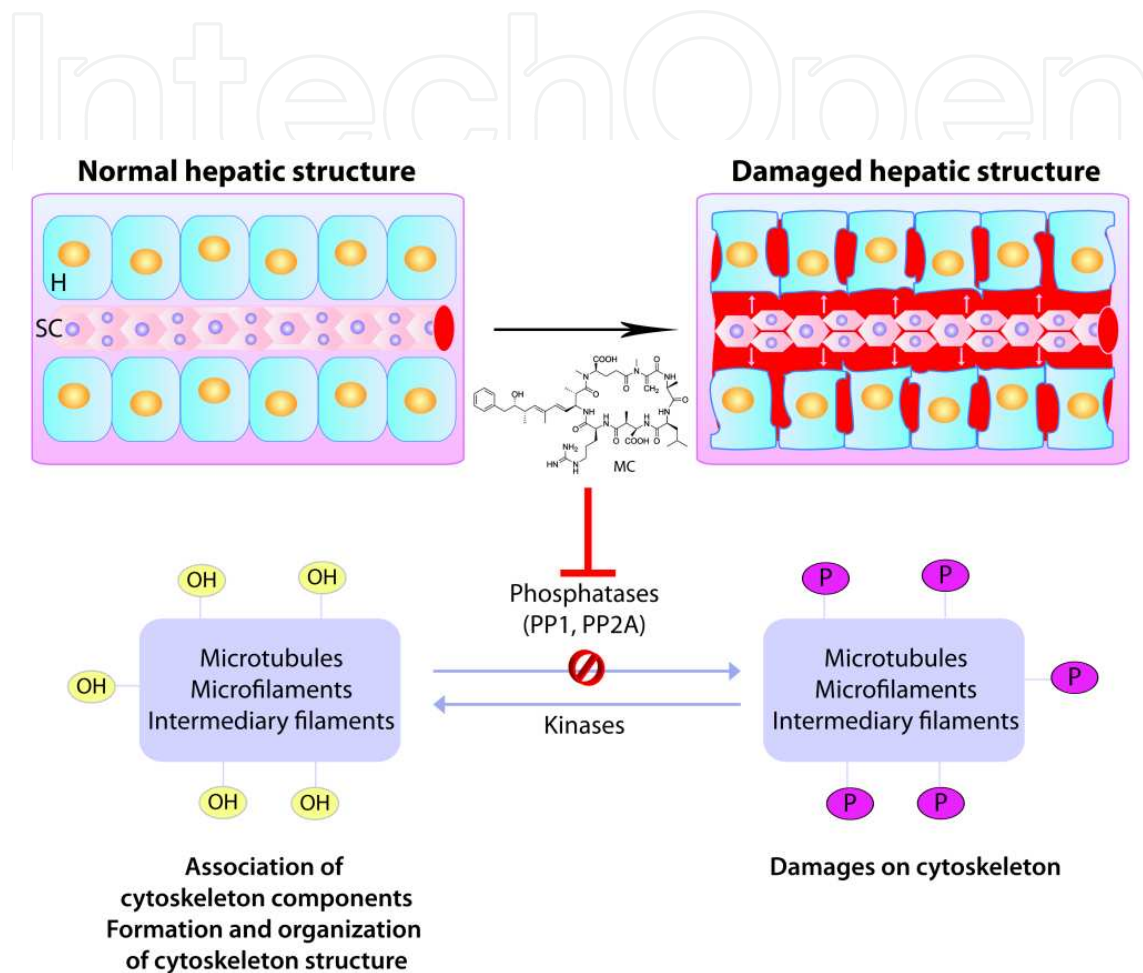

Figure 1. Representation of the effects of microcystins in cytoskeleton, namely the changes of the hepatocytes and sinusoidal capillary structure, and intrahepatic bleeding, mediated by the inhibition of protein phosphatases PP1 and PP2A (partially adapted from [5] and [6]). Legend: **H** – hepatocyte; **SC** – Sinusoidal capillary; **MC** – microcystins; **OH** – hydroxyl group; **P** – phosphate group; **PP1** – protein phosphatase 1; **PP2** – protein phosphatase 2.

Established cell lines have been considered, for a long time, as unsuitable to study the toxic effects of MCs. This was due to the observation that, comparing with primary cell lines, high amounts of MCs were required to elicit toxicity in permanent cell lines [17, 18, 19]. A proposed explanation was the fact that established cell lines lose their OATPs, which render them unable to uptake the toxin [19, 20]. Despite this fact, an increasing number of studies have demonstrated that MCs clearly induce toxic effects on several mammalian cell lines, in particular in the human hepatoma HepG2 cell line [21-24]. Indeed, it was already demonstrated that OATP transport system is preserved in the HepG2 cells [25].
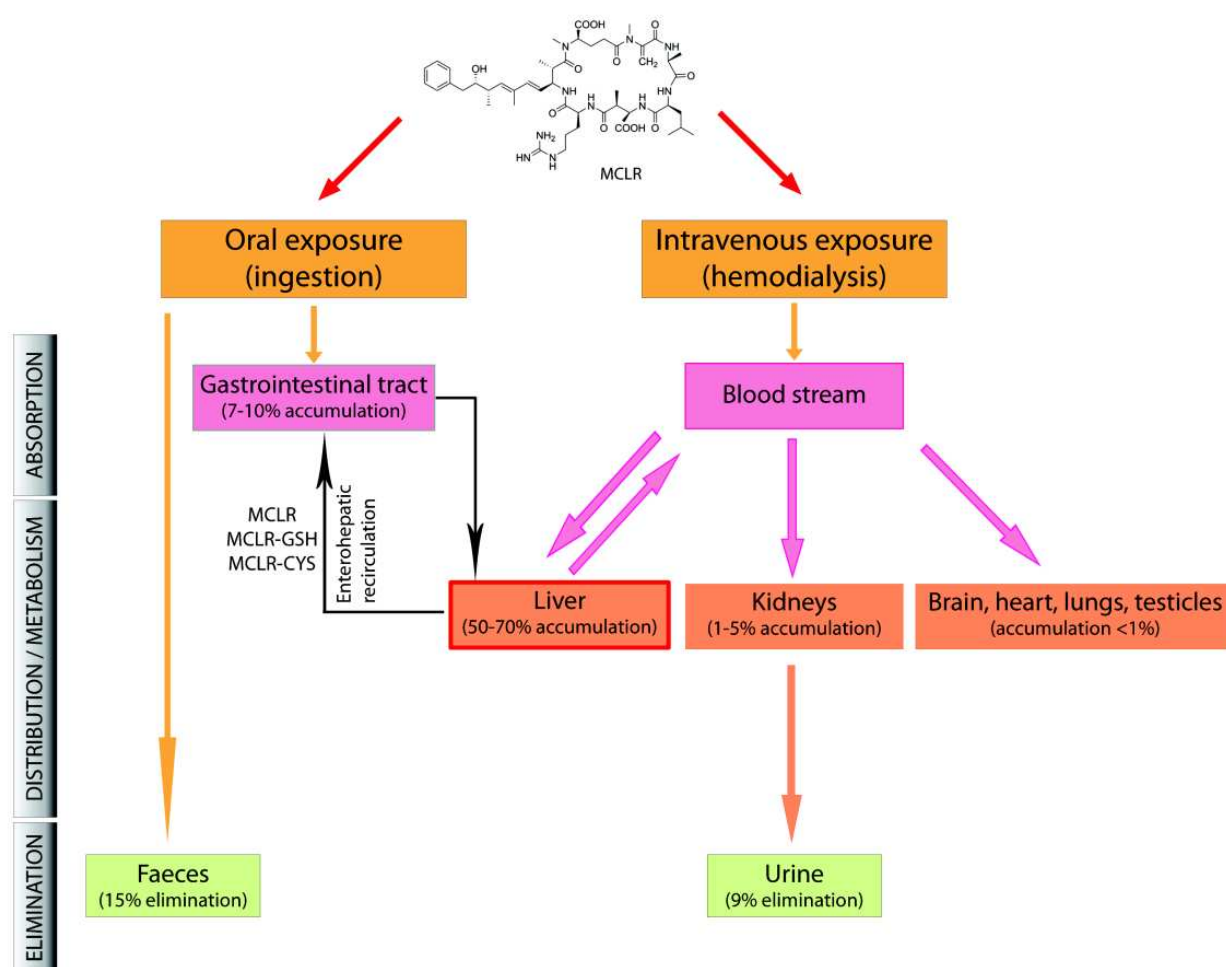
Figure 2. Schematic representation of MCLR absorption, distribution, metabolization and excretion (ADME) processes. Legend: **MCLR** – microcystin-LR; **MCLR-GSH** – microcystin-LR conjugated with glutathione; **MCLR-CYS** – microcystin-LR conjugated with cysteine.

When we started our work on MCLR toxicity in mammalian cell lines we evaluated the effects of *Microcystis aeruginosa* extracts containing MCLR in the cell viability of AML12 (*Mus musculus* hepatocytes, ATCC-CRL 2254), HepG2 (human hepatoma, ATCC-CRL 10741) and Vero-E6 (african green monkey *Cercopithecus aethiops* kidney epithelial cells, ATCC-CRL 1586) cell lines. Surprisingly, we observed that the dose-response curve of cell viability decrease induced by MCLR was quite similar in Vero-E6 and in liver-derived cell lines [26]. Since then, we have been demonstrated that MCLR induces a multiplicity of effects on Vero cells within a wide range of concentrations [26-30], according to a dose-effect relationship (Figure 3).

To our knowledge no previous study reported the expression of OATPs in Vero cell line, neither if an alternative transport system is involved in the microcystin uptake by kidney cells. The elucidation of this issue would be obviously an important contribution to the knowledge of microcystins toxicokinetics in the kidney and, consequently, an important stimulus to the investigation of microcystins toxicity in the kidneys.
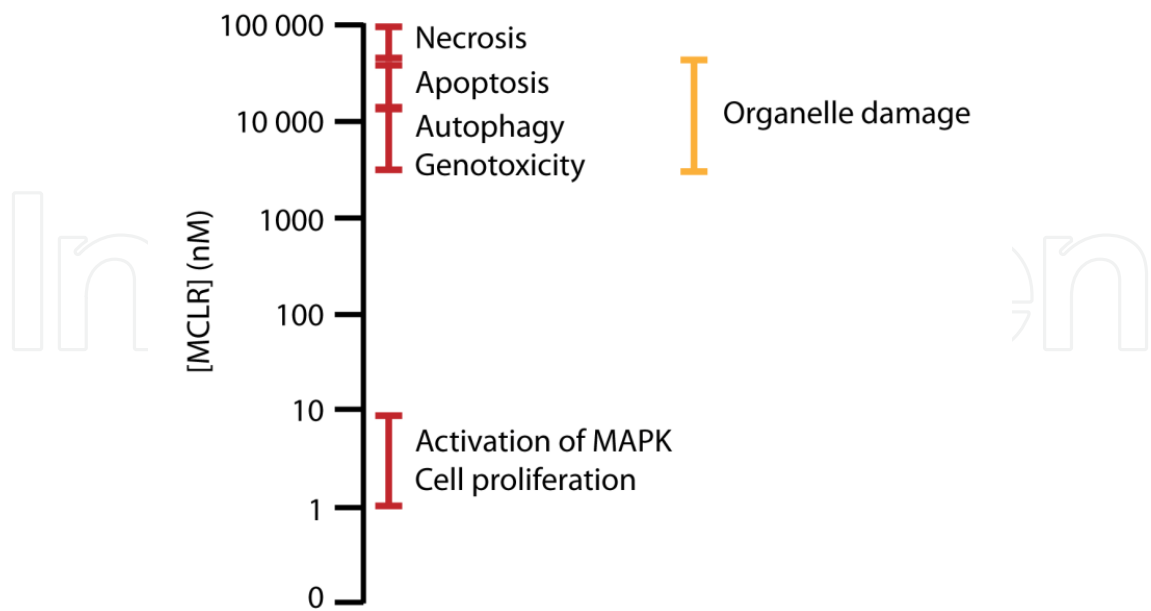
Figure 3. Dose-effect relationship of MCLR on Vero-E6 cell line.

The studies of MCs effects on cell lines often lead to contradictory results, given the fact that distinct MC toxicity endpoints (mainly cytotoxicity and genotoxicity) have been studied in diverse cell lines (and cell clones) under distinct exposure conditions (different doses-ranges, time of exposure, MCs variants, etc). In our work with Vero-E6 cells we tested MCLR (both pure toxin and from cyanobacterial extracts of *M. aeruginosa*) within a wide range of concentrations (1 nM- 200 μM), using several endpoints and methodologies (cytotoxicity, morphology, genotoxicity, protein expression). In this chapter we will summarize our results and discuss the utility of Vero-E6 cell line to evaluate the toxicological properties of MCLR.

2. Effects of MCLR in Vero-E6 cell line

2.1. Cell viability

In our studies with Vero-E6 cell line we have consistently observed that MCLR induces a concentration-dependent decrease of cell viability [26, 27, 29, 30]. This was achieved using distinct cell viability assays (MTT, Neutral Red and LDH release) and distinct toxin sources (commercially available MCLR and *M. aeruginosa* extracts). We observed that the most sensitive methods for cytotoxicity evaluation were MTT and Neutral Red assays [26, 27, 29] and that the response of pure toxin and cyanobacterial extracts was quite similar with cytotoxic thresholds within 22-50 μM of MCLR (Table 1). The MCLR-induced loss of Vero-E6 viability was attributed to apoptosis and necrosis [29, 30] as widely described for various cell types *in vitro* and *in vivo* [18, 31, 32].

2.2. Cellular organelles

In Vero-E6 cell line, exposure to MCLR (within the micromolar concentrations range) affected several cellular organelles in a concentration-dependent form (Table 1, Figure 3). In our articles, we proposed that at lower MCLR concentrations, autophagy is triggered as a survival mechanism of Vero cells, in an attempt to eliminate the toxin or/and the MCLR-induced cellular damages [29, 30]. Also, we reported the vacuolization of the Golgi apparatus and cytoplasm, effects also previously described in MCLR-exposed hepatocytes [17, 18]. The disorganization of the microfilaments and microtubules, which is one of the most commonly described cytotoxic effects of MCLR in hepatic cells [34-36], was also triggered by MCLR in Vero cells [29, 30]. The observation that MCLR induced the destructuring of the endoplasmic reticulum (ER) suggested that this organelle is involved in MCLR toxicity in Vero cell line [29, 30]. The involvement of the ER in MCLR-mediated toxicity was also previously reported in mouse kidney and liver [37]. At higher concentrations, MCLR induced the disruption of the plasma membrane, lysosomes and mitochondria of Vero cells [29, 30]. So far, studies that have used other cell types proposed mitochondria as a major target of MCLR toxicity [38-42]. However, studies with Vero cells show that mitochondria, although involved in MCLR-induced toxicity, may not be the pivotal intracellular target of the toxin [29, 30].

2.3. Genotoxicity

Based on its tumour promoter activity MCLR is classified by the International Agency for Research on Cancer as a potential human carcinogen (class 2B) [43]. In addition, some epidemiologic studies have associated the increase of human hepatocarcinoma [44, 45] and colorectal [46] cancers with the ingestion of water frequently contaminated with microcystins. However it is still unclear if, besides a tumor promoter, MCLR can also act as a tumor initiator. In fact, the potential genotoxicity of MCLR is still a matter of some controversy within the scientific community, with several authors reporting apparently contradictory results.

The hypothesis of MCLR being a genotoxic compound was mainly supported by the evidence that this toxin induces DNA damage in liver cells *in vivo*, in cultured hepatocytes and in some non-liver cell lines (revised in [47]). This data was obtained by the Comet assay, which measures DNA strand breaks that constitute primary DNA lesions with relevance for the formation of gene and chromosome mutations [48]. Several mutagenicity studies excluded the hypothesis of MCLR being a mutagen [49, 50], which was further supported by the fact that MCLR do not form adducts with DNA that would represent a pre-mutagenic lesion [51]. On the other hand, some authors reported that MCLR-induced DNA damage was a consequence of early apoptosis due to oxidative stress and not a real genotoxic effect [52]. This hypothesis was supported by the discovery that MCLR induces the formation of 8-oxo-dG, a marker of oxidative DNA damage in liver cells [51].

Chromosome damage (aneugenesis/clastogenesis) has been suggested for microcystins by an increase of the micronucleus (MN) frequency in mouse erythrocytes [49] and in the human TK6 cell line [53]. However, no effect on the micronucleus frequency has been reported for other cell types [50, 54-56] neither chromosome aberrations have been described so far [54, 57].

Some authors have also proposed that MCLR might interfere with DNA repair processes, namely the repair of gamma radiation and ultra-violet-induced DNA damage [52, 54, 57], thus increasing the genotoxic potency of those agents and contributing to their carcinogenesis.

		Toxin concentration that elicited effect at 24 h (extension of effect)		Methods	References
	Effects	Pure MCLR	Cyanobacteria extract w/ MCLR		
Cytotoxicity (cell viability decrease)		25 – 200 µM (52-82%)	22-175 µM (55-58%)	MTT assay	[27]
		–	30 µM - 150 µM (35-67%)	MTT assay	[29]
		–	30 µM - 150 µM (26-79%)	Neutral Red assay	[29]
		50 -100 µM (48-66%)	50 -100 µM (80-82%)	Neutral Red assay	[30]
		200 µM (55%)	88-175 µM (38-29%)	LDH release	[27]
Cellular organelles damages and /or changes	Autophagosome induction	–	5-12 µM	TEM (Immuno)fluorescence microscopy Tunnel assay	[29, 30]
	Endoplasmic reticulum		5-50 µM		
	Cytoskeleton damages		12-50 µM		
	Lysosome rupture		20-50 µM		
	Mitochondria damages				
Apoptosis			20-50 µM (13-27%)		
Necrosis			12-100 µM (10-25%)		
Genotoxicity		Within micromolar range	20 and 40 µM (3.4 and 4.1 fold)	Micronucleus assay	[26, 58]
ERK1/2 activation		5-5000 nM (2.8–4 fold)	5-5000 nM (1.7–3.7 fold)	Western Blot	[28]
Cell proliferation		1– 10 nM (1.5-2.1 fold)	-	BrdU incorporation assay	

Table 1. Effects induced by pure MCLR and toxic *M. aeruginosa* extracts in Vero-E6 cell line: effective toxin concentrations, effect extension and methods used in their evaluation.

In our early studies with the Vero-E6 cell line we demonstrated that cyanobacterial extracts from a MCLR-producer cyanobacterial strain increased the frequency of micronuclei at non-cytotoxic concentrations [26]. Afterwards, we confirmed this observation using pure MCLR [58, submitted for publication]. In this study, we found that MCLR induces the micronucleation of Vero-E6 and human hepatoma HepG2 cell lines. In order to disclose the mechanism underlying micronuclei formation we used the centromere labelling Fluorescent in situ Hybridization technique in HepG2 exposed cells to MCLR (since there are no commercial centomere probes for monkey cells) and we observed that MCLR induced both centromere-positive and centromere-negative micronuclei [58]. Our data, together with those from other works, suggests that MCLR genotoxicity occurs indirectly by both clastogenic and aneugenic mechanisms: the first, possibly through oxidative stress; the second, perhaps through damages on the mitotic spindle, induced by the inhibition of PP1/PP2A. Since clastogenesis and aneugenesis have been associated with human cancer development [59] it can be hypothesized that both underlie the carcinogenic activity of MCLR. Moreover, the confirmation of genotoxic activity of MCLR *in vivo* is of major importance for regulatory purposes because a safe level could not be applied to clastogens conversely to aneugens [59, 60]. This is fundamental for the

prevention of the risk of exposure of human populations to water contaminated with toxic cyanobacteria.

In figure 4 we summarize the proposed effects/mechanisms of MCLR genotoxicity, based on the previous reports from other authors and our own contribution.
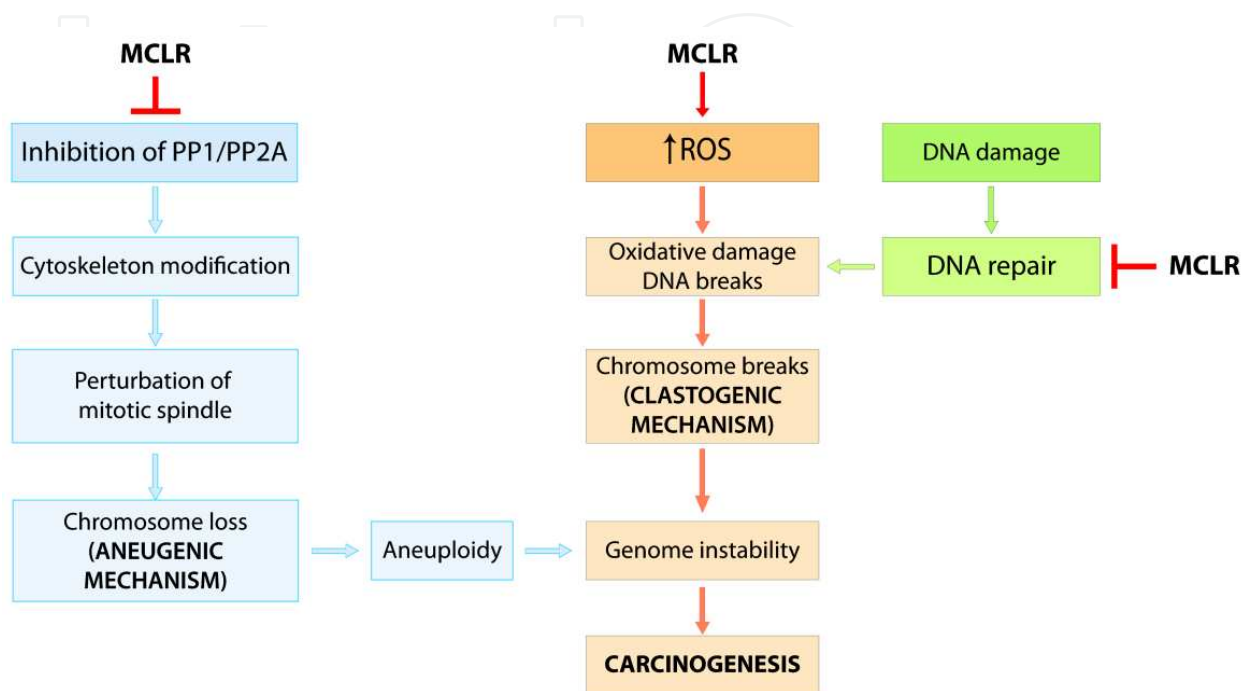

Figure 4. Representation of possible mechanisms of genotoxicity induced by MCLR. Legend: **MCLR** – microcystin-LR; **PP1** – protein phosphatase 1; **PP2** – protein phosphatase 2; **ROS** – reactive oxygen species.

2.4. Cell proliferation

It is generally assumed that MCLR is a potent tumor promoter. This assumption is based on rodent carcinogenicity studies which revealed that MCLR is able to induce cellular transformation of rat liver [61] and mouse skin [62] of animals previously exposed to a genotoxic agent. However, the mechanisms underlying MCLR-induced tumor promotion are still unknown. It has been suggested that this activity is mediated by the inhibition of serine/threonine phosphatase PP1 and PP2A, given their role on the regulation of cellular division and proliferation, namely through the activation of Mitogen-Activated Protein Kinases (MAPK) [63]. MAPKs are involved in signaling pathways that regulate many cellular processes through phosphorylation cascades, in particular the Ras-Raf-MEK1/2-ERK1/2 cascade, with a key role in cellular proliferation and being regulated by several types of phosphatases including the serine/threonine phosphatases PP1 and PP2A [64]. The Ras-Raf-MEK1/2-ERK1/2 cascade is activated by growth factors and mitogenic agents (Figure 5). They bind to tyrosine kinase membrane receptors (RTK), which activate the membrane G-protein GTPase, the recruitment and activation of Raf protein and the subsequent phosphorylation cascade of ERK1/2 pathway [65]. The activated (phosphorylated) forms of ERK1/2 are translocated to the cell nucleus, inducing

the activation of transcription factors such as c-Fos and c-Jun thus triggering cell proliferation [64]. The fact that Ras-Raf-MEK-ERK cascade is regulated by several types of phosphatases including the protein serine/threonine phosphatases PP2A [64, 66] supports the hypothesis that, by inhibiting PP2A, MCLR deregulates the ERK1/2 pathway and promotes cell proliferation (Figure 5).
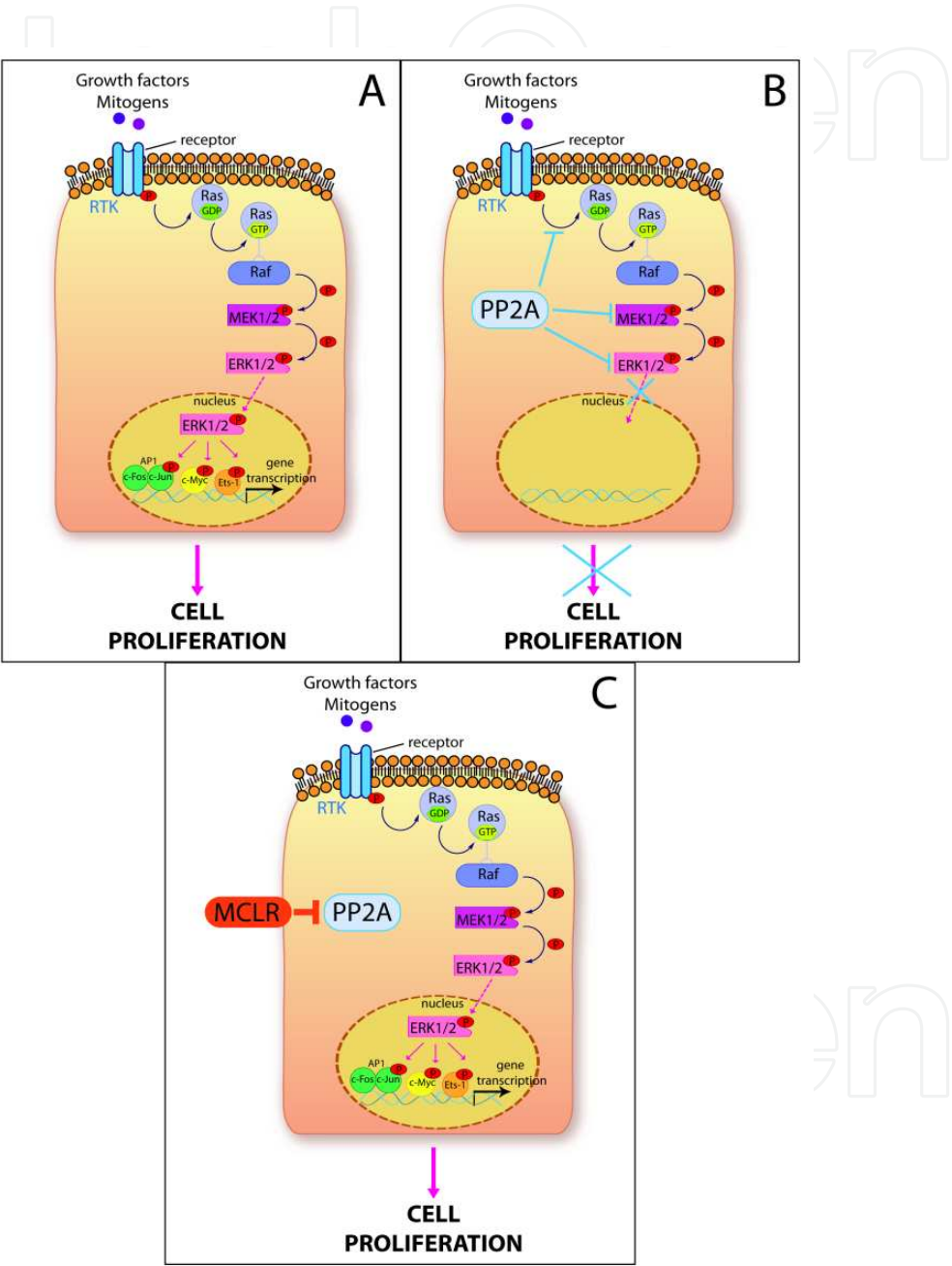

Figure 5. The role of protein phosphatase PP2A in the regulation of the signaling pathway Ras-Raf-MEK1/2-ERK1/2. Partly based on Kolch [67] and Junttila et al [64]. Legend: **RTK** (*tyrosine kinase receptor*); **Ras** (GTPase); **Raf** (*MAP kinase kinase kinase*); **GDP** (guanosine diphosphate); **GTP** (guanosine triphosphate); **P** (phosphate group); **MEK** (*MAP kinase kinase*); **ERK** (*extracellular-signal-regulated kinase*); **AP1** (*activator protein-1*); **c-Fos, c-Jun, c-Myc, ETS-1** (transcription factors); **PP2A** (type 2A protein phosphatase).↓ Activation, ⊥ Inhibition.

Few studies support this hypothesis: (1) Li et al [68] reported the activation of proto-oncogenes c-jun, c-fos and c-myc by a cyanobacterial extract containing microcystins in rat liver, kidney and testis; (2) Zhu et al [69] demonstrated that MCLR induces the transformation of immortalized colorectal crypt cells through the constitutive activation of AKT and MAPK (p38 and JNK) cascades. Our team has evaluated the effect of MCLR in Vero-E6 cell line proliferation through the BrdU incorporation assay that evaluates the G1/S transition in cell cycle [28]. We showed that MCLR (1 to 10 nM) induces a significant increase in Vero cells proliferation with a maximum of 2.2 fold increase at 5 nM [28]. We further analyzed the expression of MAPK (ERK1/2, JNK and p38) by Western-blot and concluded that MCLR stimulates Vero cells proliferation by the activation of the ERK1/2 signaling pathway [28]. These results emphasize the importance to confirm the impact of MCLR on tumor promotion *in vivo*, in particular at kidney level.

3. Comparison of MCLR-induced toxicity in kidney cell lines

The effects of microcystins in kidney cell lines, namely in Vero cells, have been barely evaluated. Thompson et al [70] reported that cyanobacteria extracts containing up to 10 μM of MCLR did not interfere with the morphology and LDH release of Vero cells. Chong et al. [21] also did not found changes in Vero cells viability (evaluated by the MTT assay) after exposure to concentrations up to 37.5 μM of pure MCLR during 24–96 h. Grabow et al. [71] described cytopathogenic effects (rounding and disintegration of cells) of Vero cells induced by *M. aeruginosa* extracts containing 500 μM of MCLR. The results from our studies show a higher sensitivity of Vero-E6 cell line to MCLR than those previously reported data for Vero cells (Table 1).

Additionally, in a previous study we also observed that MCLR induce cytotoxic effects in the Madin-Darbin canine kidney cell line (MDCK – ATCC CCL-34) [72]. However, the sensitivity of this cell line was lower than that of Vero cells. In fact, while significant reduction of Vero cells viability occurs above 25 μM of MCLR (Table 1), only 30% decrease in MDCK cell viability was observed after exposure to 100 μM of MCLR, using the Neutral Red Assay [72].

Effects of MCLR on cell morphology and ultrastructure were previously evaluated in the rat renal epithelial cell line NRK-52E (ATCC-CRL 1571) in studies developed in 1990's decade. Wickstrom et al. [73] found that MCLR affects the cytoskeleton components namely the microtubules, intermediate filaments and microfilaments in a similar way to that observed in hepatocytes. However, the renal cell line required a 100-fold higher concentration (more than 100 μM) and prolonged time of exposure comparing to primary hepatocytes [73]. Reports from Khan et al. [17, 74] also demonstrated the collapse and condensation of cystoskeleton elements induced by MCLR (133 μM) on NRK-52E cell line.

The differences reported in the above mentioned distinct studies might be explained by differences in experimental design, such as the use of toxins from different sources (pure or crude extracts), applied in different dosages and tested by different endpoints of toxicity. Besides, the use of different clones of Vero cells (often not mentioned in the papers) may also

justify the distinct sensitivities observed among several authors. Further studies would also be required to conclude if the effects of MCLR on kidney cells could be species-dependent.

Overall, the cytotoxic, morphological and ultrastructural effects of MCLR on Vero-E6 cell line reported by us are quite similar to those reported for other cell lines. However, we observed these effects at lower concentrations, which might suggest an eventual higher sensitivity of Vero-E6 cell line comparing to other kidney cell lines.

4. Is Vero-E6 cell line a suitable model to study toxicological properties of microcystins?

The assessment of kidney injury/dysfunction *in vivo* presents some complexity given the fact that the kidney is constituted by over 20 different cell types exhibiting distinct morphologies and functions and, consequently, diverse responses to toxic compounds [75, 76]. For this reason, *in vitro* models are useful tools to assess specific nephrotoxic effects on specific cell types [75]. Renal epithelial cell lines, in particular, present some advantages: there are several well characterized commercially available clones, they are easy to grow and manipulate and some of them retain basic functions of their original ancestors from kidney *in vivo* [76]. Specific biochemical, morphological and functional markers such as transepithelial resistance and transport, might therefore be used as endpoints of nephrotoxicity *in vitro* [75-77].

Vero-E6 monkey kidney cell line has been widely used on toxicology, virology and pharmacology research, as well as, on the production of vaccines and diagnostic reagents [78]. In particular, these cells have been used as model for assays to evaluate the toxicity of compounds of different nature, either chemical or microbial toxins. The chemical substances tested include carbamazepine [79]; triclosan [80]; lead nitrate [81]; pentachlorophenol and rotenone [82], where the Vero cell line revealed to be one of the most sensitive model used in these studies. These cells have also been validated as a cellular model for other microbial toxins such as diphtheria toxin, a polypeptide with 535 a.a. [83] and Shiga-like toxins, a protein of enterohemorrhagic *Escherichia coli* [84]. The sensitivity of Vero cell line to another cyanobacterial toxin, cylindrospermopsin, was also reported although, such as MCs, the mechanism of toxin uptake by this cell line remains to clarify [85, 86].

In our studies on the effects of MCLR on Vero-E6 cell line we did not evaluate any specific nephrotoxicity marker. Instead, we evaluated the basal toxicity of MCLR, that is, the effects that might be common to all cell types [87].

Using diverse methodologies including standard methods to evaluate cytotoxicity (Neutral Red, MTT and Lactate Dehydrogenase release) and genotoxicity (Micronucleus and Comet assay) we observed that MCLR induces a multiplicity of effects on Vero-E6 cells at distinct levels: cellular morphology/ultrastructure, cell viability/death, MAPK expression and genotoxicity (as referred in section 2). The type and extension of these effects were highly dependent of toxin concentration and, generally, a dose-response relation could be established: for a dose range of 1-10 nM, MCLR stimulates cell proliferation through the activation of the mitogen

activated protein kinase ERK1/2 signalling pathway; however, within the µM range, MCLR triggers a variety of effects in almost all cell compartments, from genotoxicity (induction of micronuclei) and autophagy to apoptotic and necrotic cell death. Therefore, MCLR induces a dual effect on the Vero-E6 cell line: at low doses it stimulates the cell growth but at high doses it induces a decrease in viability and cell death (Figure 6).
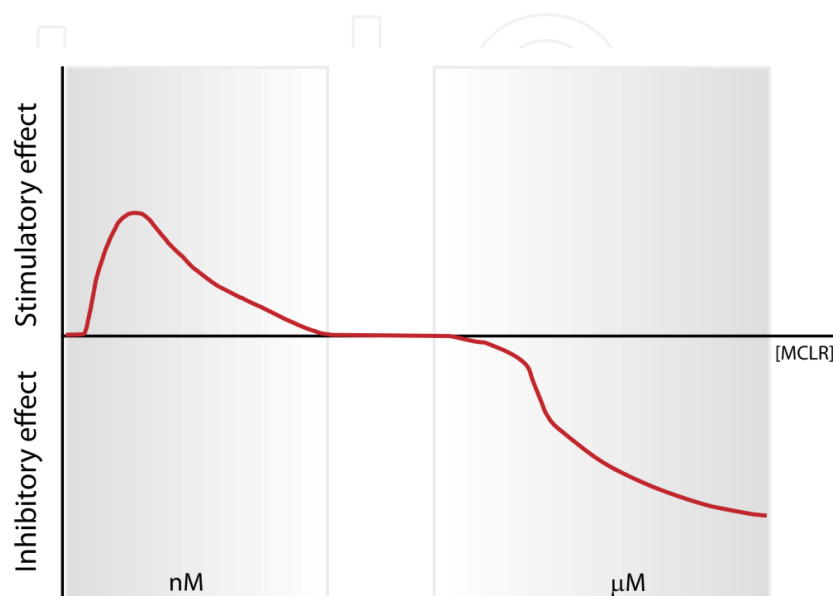

Figure 6. Hormetic dose-response curves of MCLR on the growth of Vero-E6 cells.

This duality of low-dose growth stimulation *vs* high-dose growth inhibition was commented by Li et al [88] as a characteristic hormetic dose-response phenomenon, such as they observed for the low-dose PP2A stimulation *vs* high-dose PP2A inhibition by MCLR. However, additional experiments would be required to support the hypothesis of MCLR exhibiting a hormesis dose-response relation.

Another aspect we would like to underline is that we can attribute with high certainty the observed responses of Vero-E6 cell line to MCLR exposure. The results obtained with toxic cyanobacterial extracts are often questioned due to the uncertainty of cyanobacteria extracts composition and the putative interference between cyanotoxins and other cyanobacterial bioactive compounds [89]. In our studies, we tested two sources of MCLR: cyanobacterial extracts from MCLR-producers (strains of *Microcystis aeruginosa* LMECYA 7, LMECYA 110 and LMECYA 113) [90] and commercially available pure MCLR. We have included in our experiments two types of controls: culture medium when using pure toxin and a cyanobacterial extract from a non toxic *Microcystis aeruginosa* strain (LMECYA 127) when using the toxic cyanobacterial extract. All the strains of *M. aeruginosa* were phylogenetically close related [91] and the ability (or inability) to produce MCLR was previously tested by several analytical methodologies [92]. According to our results on cytotoxicity, genotoxicity and ERK1/2 (MAPK) activation, all toxin solutions induced statistically significant responses in relation to respective controls. Besides, the type and extension of effects were quite similar irrespectively of the toxin

source (Table 1) and the results were repeatable after performing independent experiments. In this sense we conclude that the observed effects were induced specifically by MCLR.

Usually it is assumed that high amounts of MCLR are required to elicit any effect on cell lines comparing to primary cells [19]. This is true, but it also depends on the effects that are being evaluated. In fact, cytotoxic MCLR concentrations for Vero cells were found within the range of micromolar (µg/mL). However, the lowest concentration that elicited an effect (cell proliferation) on Vero-E6 cell line was 1 nM (µg/L) [28]. Many of the effects induced by MCLR in Vero-E6 cell line were quite similar to those induced in the human hepatoma cell line (HepG2) at similar dose-ranges. This applies to our data on MCLR genotoxicity on distinct cell models [58] as well as on reported data from other authors [22, 47]. In fact, HepG2 cell line has been considered a suitable model to evaluate the effects of MCLR on liver-derived cells, which similarly might be applicable to Vero-E6 cell line regarding kidney-derived cells.

Considering all these aspects, we propose that Vero-E6 cell line is an appropriate *in vitro* cell model to evaluate the basal toxicity of MCLR, although proper assessment of the specificity, sensitivity, accuracy, among other parameters, will be required to validate this model. Additionally, the elucidation of the MCLR uptake mechanism (OATPs or other transport system), as well as the immunolocalization of MCLR within Vero cells, could be used to confirm the access of this toxin to a kidney-derived cell line. Ultimately, further experiments aiming at evaluate the effects of MCLR on specific kidney epithelial cells properties/nephrotoxic endpoints constitute a fundamental step in order to demonstrate the utility of Vero-E6 cell line as an *in vitro* model to investigate the nephrotoxic mechanisms of MCLR.

5. Concluding remarks

Despite the gaps in knowledge regarding the toxicological properties of microcystins, an increasing number of recent publications emphasize the need to carefully evaluate their effects on other organs besides the liver, particularly their carcinogenicity. The adverse effects of MCLR in distinct organs is an important issue for risk assessment, because the guideline value for MC in drinking water (1 nM) is still a provisional value, based on limited toxicological data [93].

The exposure to low doses of MCLR corresponds to the most realistic kidney intoxication scenario, considering that it is not the main target organ of this toxin. However, the role of kidneys in toxin elimination might lead to the exposure of kidney cells to a low internal dose that can be biologically effective in the induction of nephrotoxic effects. Besides, the chronic adverse effects of microcystins on kidneys, specially the potential tumorigenic/carcinogenic effects, may assume a particular importance in populations exposed to water persistently contaminated with toxic cyanobacteria.

Although further studies will be required to recognize the epithelial monkey kidney-derived Vero-E6 cell line as a nephrotoxicity model, we propose that Vero-E6 cell line constitutes a valuable in vitro model to evaluate the basal toxicity of MCLR and to study the mechanisms of MCLR toxicity.

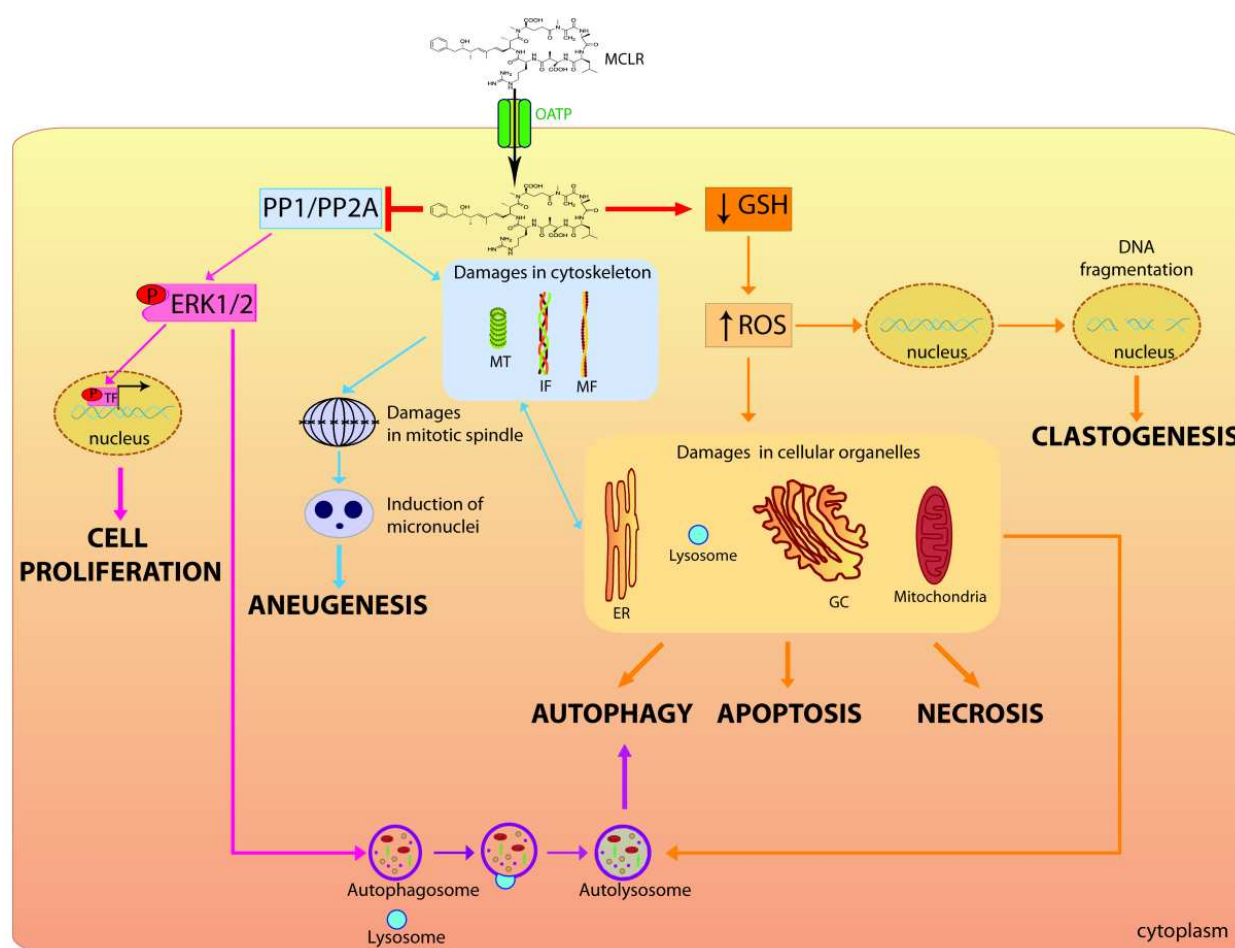In figure 7 we summarize the effects induced by MCLR on Vero-E6 cell line.

Figure 7. Effects and mechanisms of toxicity of MCLR on Vero-E6 cell model. Legend: **OATP** (organic anion transport-ers polypeptide); **PP1/PP2A** (protein phosphatases PP1 and PP2A); **P** (phosphate group); **ERK1/2** (extracellular-signal-regulated kinase); **TF** (transcription factors); **MT** (microtubules); **IF** (intermediary filaments); **MF** (microfilaments); **GSH** (reduced glutathione); **ROS** (reactive oxygen species); **ER** (endoplasmic reticulum); **GC** (golgi complex). ↓ Activation, ⊥ Inhibition.

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