Can LASSBio 596 and dexamethasone treat acute lung and liver inflammation induced by microcystin-LR?


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Abstract

The treatment of microcystin-LR (MCYST-LR)-induced lung inflammation has never been reported. Hence, LASSBio 596, an anti-inflammatory drug candidate, designed as a symbiotic agent that modulates TNF-α levels and inhibits phosphodiesterase types 4 and 5, or dexamethasone were tested in this condition. Swiss mice were intraperitoneally (i.p.) injected with 60 μl of saline (CTRL) or a sub-lethal dose of MCYST-LR (40 mg/kg). 6 h later they were treated (i.p.) with saline (TOX), LASSBio 596 (10 mg/kg, L596), or dexamethasone (1 mg/kg, 0.1 mL, DEXA). 8 h after MCYST-LR injection, pulmonary mechanics were determined, and lungs and livers prepared for histopathology, biochemical analysis and quantification of MCYST-LR. TOX showed significantly higher lung impedance than CTRL and L596, which were similar. DEXA could only partially block the mechanical alterations. In both TOX and DEXA alveolar collapse and inflammatory cell influx were higher than in CTRL and L596, being LASSBio 596 more effective than dexamethasone. TOX showed oxidative stress that was not present in CTRL and L596, while DEXA was partially efficient. MCYST-LR was detected in the livers of all mice receiving MCYST-LR and no recovery was apparent. In conclusion, LASSBio 596 was more efficient than dexamethasone in reducing the pulmonary functional impairment induced by MCYST-LR.

1 Introduction

In the last years cyanobacterial blooms increased all over the world. Because this phenomenon also takes place in aquatic ecosystems used as drinking water supplies and these cyanobacteria produce cyanotoxins (e.g., microcystins), a public health concern results (Chorus and Bartram, 1999). Humans may be exposed to cyanotoxins through several routes: the oral one is by far the most important. However, dermal, inhalation and parenteral routes may also occur (Funari and Testai, 2008).

In 1988 a possible association between a cyanobacterial bloom and 88 deaths among 2000 intoxicated people was described (Teixeira et al., 1993). In 1990 two recruits in England developed severe pneumonia after contact with water containing toxic Microcystis aeruginosa (Turner et al., 1990). Microcystins can reach the lung, and their distribution after oral, intraperitoneal and intratracheal administrations has been described (Ito et al., 2000, 2001). Finally, in February 1996 a death was recorded among patients undergoing routine renal dialysis with water contaminated with microcystins; the remaining patients...
developed acute liver failure, and 52 died by December 1996. These were the first confirmed human deaths related to microcystin intoxication (Barreto et al., 1996; Azevedo et al., 2002).

Microcystins are the cyanotoxins most frequently found all over the world. In vertebrate animals, the main targets of microcystins are the hepatic cells, where they irreversibly inhibit protein phosphatases (PP) of the serine/threonine family, especially PP1 and PP2A. Acutely, cytoskeleton disarrangement of hepatocytes follows, leading to hepatic failure. We previously reported a rapid and continued lung inflammatory process with interstitial edema, alveolar collapse and recruitment of inflammatory cells in Swiss mice injected with a sub-lethal intraperitoneal dose of an aqueous extract containing MCYST-LR, the most potent toxin (Picanço et al., 2004). Recently we observed pulmonary mechanical impairment in Swiss mice that received purified MCYST-LR (Soares et al., 2007). Additionally, the inflammatory process can damage the lung tissue by means of reactive oxygen species (ROS) produced by activated defense cells, such as neutrophils, monocytes and macrophages (Moreno et al., 2005). There are no reports in the literature concerning the treatment of the pulmonary damage induced by microcystin-LR.

Thus, we aimed to evaluate whether LASSBio 596 or dexamethasone could protect the lung and liver against the toxic actions of microcystin-LR. For such purpose lung mechanics and biochemical analyses, as well as lung and liver histology were evaluated in Swiss mice.

2. Materials and methods

2.1. Animals

Swiss male mice (6–7 weeks of age) were purchased from FIOCRUZ (Fundação Oswaldo Cruz, Rio de Janeiro, Brazil). The animals were housed in plastic cages with absorbent bedding material and maintained on a 12 h daylight cycle. Food and water were provided ad libitum.

All animals received humane care in compliance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences, USA. The experiments were approved by the Ethics Committee of the Health Sciences Center, Federal University of Rio de Janeiro (Protocol IBCCF 012).

2.2. Preparation

Forty mice (35–40 g) were randomly divided into 4 groups. They were initially intraperitoneally (i.p.) injected with 60 μl of either sterile saline solution (0.9% NaCl, CTRL, n = 9) or a sub-lethal dose of MCYST-LR (40 μg/kg i.p., purified material kindly provided by Professor Wayne Carmichael, Wright State University, Dayton, OH, USA). After 6 h CTRL animals received 200 μl (i.p.) of sterile saline solution, while those injected with MCYST-LR were injected with 200 μl (i.p.) of either sterile saline solution (TOX, n = 9), LASSBio 596 (10 mg/kg, LS96, n = 8), or dexamethasone (1 mg/kg, DEXA, n = 10). Initially each group comprised 10 animals; four animals died along the experiment.

2.3. Pulmonary mechanics

Eight hours after MCYST-LR injection, i.e., 2 h after treatment, the animals were sedated with diazepam (1 mg i.p.), anesthetized with pentobarbital sodium (20 mg kg body weight⁻¹ i.p.), tracheotomized, and a snugly fitting cannula (0.8 mm id) was introduced into the trachea. Then, the animals were paralyzed with pancuronium bromide (0.1 mg/kg i.v.), and a constant-flow ventilator (Samay VR15, Universidad de la Republica, Montevideo, Uruguay) provided artificial ventilation with a frequency of 100 breaths/min, a tidal volume of 0.2 ml, flow of 1 ml/s, and positive end-expiratory pressure amounting to 2 cm H2O. The anterior chest wall was surgically removed.

A pneumotachograph (1.5 mm ID, length = 4.2 cm, distance between side ports = 2.1 cm) (Mortola and Noworaj, 1983) was connected to the tracheal cannula for the measurements of airflow (V). Lung volume (Vbar) was determined by digital integration of the flow signal. Tracheal pressure was measured with a Validyne MP-45 differential pressure transducer (Engineering Corp, Northridge, CA, USA). The flow resistance of the equipment (Req), tracheal cannula included, was constant up to flow rates of 26 ml s⁻¹ and amounted to 0.12 cm H2O ml⁻¹ s. Equipment resistive pressure (=Req.V) was subtracted from pulmonary resistive pressure so that the present results represent intrinsic values. All signals were conditioned and amplified in a Beckman type R Dynograph (Schiller Park, IL, USA). Flow and pressure signals were then passed through 8-pole Bessel filters (902LPF, Frequency Devices, Haverhill, MA, USA) with the corner frequency set at 100 Hz, sampled at 200 Hz with a 12-bit analog-to-digital converter (DT2801A, Data Translation, Marlboro, MA, USA), and stored on a microcomputer. All data were collected using LABDAT software (RHT-InfoData Inc., Montreal, QC, Canada).

Lung resistive (ΔP1) and viscoelastic/inhomogeneous (ΔP2) pressures, total pressure drop (ΔPtot = ΔP1 + ΔP2), static elastance (Est), and elastic component of viscoelasticity (ΔE) were computed by the end-inflation occlusion method (Bates et al., 1985, 1988). Briefly, ΔP1 selectively reflects airway resistance in normal animals and humans and ΔP2 reflects stress relaxation, or viscoelastic properties of the lung, together with a tiny contribution of time constant inequalities (Bates et al., 1988; Saldiva et al., 1992). Lung static (Est) elastance was calculated by dividing Pel (elastic recoil pressure of the lung) by Vbar. ΔE was calculated as the difference between static and dynamic elastances (Bates et al., 1985, 1988). Pulmonary mechanics were measured 10–15 times in each animal.

All data were analyzed using ANADAT data analysis software (RHT-InfoData Inc., Montreal, QC, Canada). The duration of the experiments approximated 30 min.

2.4. Histological analysis

A lower longitudinal laparotomy was done immediately after the determination of pulmonary mechanics, and heparin (1000 IU) was intravenously injected. The trachea
was clamped at end-expiration, and the abdominal aorta and vena cava were sectioned, yielding a massive hemorrhage that quickly euthanized the animals. Then the lungs were removed en bloc. Two investigators, who were unaware of the origin of the coded material, examined the samples microscopically.

The lungs were quick-frozen by immersion in liquid nitrogen, fixed with Carnoy’s solution, and embedded in paraffin. Four-μm-thick slices were obtained by means of a microtome and stained with hematoxylin and eosin (HE). Morphometric analysis was performed with an integrating eyepiece with a coherent system with 100 points and 50 lines coupled to a conventional light microscope (Axioplan, Zeiss, Oberkochen, Germany). The point-counting technique was used across 10 random non-coincident microscopic fields to evaluate the fraction area of collapsed alveoli (×200) and the amount of polymorphonuclear cells (PMN) expressed as cells/pulmonary tissue area (×1000) (Gundersen et al., 1988).

The livers were removed right after lung exeresis, fixed in buffered formaldehyde (10%) and embedded in paraffin. Four-μm-thick slices were stained with HE. A pathologist, who was unaware of the origin of the material, examined the samples at magnifications of ×100 and ×400.

2.5. Biochemical analyses

Another 24 mice (35–40 g) were randomly divided as aforementioned. The left lung was used for determination of total protein content by the method of Bradford (1976) and inflammation and oxidative stress analyses. The right lung and the liver of each animal were isolated for free MCYST-LR analysis by enzyme-linked immunosorbent assay (ELISA).

2.5.1. Myeloperoxidase (MPO)

Inflammatory changes were examined by MPO activity in the supernatant of lung homogenates. Absorbencies were determined at 655 nm using a plate reader (Model 550, Bio-Rad, Hercules, CA, USA) (Suzuki et al., 1983).

2.5.2. Lipid peroxidation, superoxide dismutase (SOD) and catalase (CAT)

The thiobarbituric acid-reactive substances (TBARS) method analyzed malondialdehyde (MDA) products during an acid-heating reaction (Draper and Hadley, 1990). TBARS levels were determined at 532 nm and expressed as MDA equivalents (nM/mg protein). SOD activity was assayed by measuring inhibition of adrenaline auto-oxidation as absorbance at 480 nm and was expressed as SOD equivalents (U/mg protein) (Bannister and Calabrese, 1987). CAT activity was measured by the rate of decrease in hydrogen peroxide concentration at 240 nm and was expressed as CAT equivalents (U/mg protein) (Aebi, 1984). A spectrophotometer was used in all determinations (Ultrspec 2100 pro, Amersham-Biosciences, Buckinghamshire, UK).

2.6. Determination of microcystin-LR

This assay identifies free microcystin by a combination of secondary anti-IgG antibodies and primary anti-MCYS

Fig. 1. Pressures used to overcome resistances in mice lung. ΔP1, resistive pressure; ΔP2, pressure dissipated to overcome viscoelastic/inhomogeneous mechanical components; ΔPtot, total pressure spent against viscous and viscoelastic components. CTRL, TOX, L596, and DEXA, animals that received saline, microcystin-LR, microcystin-LR plus LASSBio 596, and microcystin-LR plus dexamethasone, respectively. Bars are mean ± SEM of 8–10 animals in each group (10–15 determinations per animal). Different letters indicate significantly different values (p < 0.05).

SigmaStat 3.11 statistical software (SYSTAT, Chicago, IL, USA) was used. The normality of the data (Kolmogorov–Smirnov test with Lilliefors’ correction) and the homogeneity of variances (Levene median test) were tested. Since in all instances both conditions were satisfied, one-way
ANOVA followed by Student-Newman-Keuls test was used to assess differences among groups, when required. The morphometric parameters underwent an arcsine transformation and, then, were compared. In all instances the significance level was set at 5% ($p < 0.05$).

3. Results

3.1. Pulmonary mechanics

There was no statistically significant difference in flow and inspired volume among the groups. All mechanical parameters were higher in TOX than in CTRL. L596 and CTRL produced similar data, but in DEXA increases in $\Delta P1$ and $\Delta P\text{tot}$ like those in TOX were detected (Figs. 1 and 2).

3.2. Lung histology

Fig. 3 shows lung parenchyma damaged in TOX, L596 and DEXA groups. Discrete edema and thickening of alveolar septa were present in TOX and DEXA. Influx of PMN cells was higher in TOX and DEXA compared to CTRL and L596 groups. Collapsed areas were found in animals injected with microcystin-LR, progressively decreasing from TOX to L596 (Fig. 4).

3.3. Biochemical analyses

MPO, TBARS and CAT were significantly higher in TOX than in the other three groups that did not differ among themselves, as depicted in Fig. 5. As also shown in Fig. 5, SOD activity was higher in CTRL and L596 groups than in TOX and DEXA; the latter were similar.

3.4. Determination of microcystin-LR

Microcystin was not detected in lung homogenates, but free MCYST-LR was present in the livers of TOX, L596 and DEXA animals. MCYST-LR contents did not differ among these three groups (Fig. 6).

3.5. Liver histology

Fig. 7 shows that mice from TOX, L596 and DEXA displayed a complete loss of liver architecture with necrosis, inflammation, a high degree of binucleated hepatocytes, cytoplasmatic vacuolization, dilated sinusoidal spaces and hyalinization.

4. Discussion

We compared the effects of LASSBio 596 and dexamethasone on acute lung inflammation induced by microcystin-LR in Swiss mice. The former compound was more powerful in avoiding increases in in vivo pulmonary impedance, alveolar collapse, PMN influx and oxidative stress than the latter. Free MCYST-LR could not be detected in mice lungs, but was present in the liver of animals that received the toxin, which did not evidence any sign of liver recovery.

Our model of intraperitoneally injected MCYST-LR could partially mimic human contact with microcystins. Animal and human exposure to low concentrations of microcystins is much more frequent than lethal events, damaging many organs (Nobre et al., 2003; Kujbida et al., 2008). In this context, we had observed histological and functional lung alterations (Picanço et al., 2004; Soares et al., 2007). Thus, our mice were exposed to a sub-lethal dose of MCYST-LR (40 $\mu$g/kg).

In our previous study (Soares et al., 2007) mechanical and histological impairment occurred as soon as 2 h after intraperitoneal administration of MCYST-LR in mice. These changes persisted for 4 days and the highest percentage of collapsed areas was detected at 8 h after MCYST-LR. In 2003, it was reported that LASSBio 596 administered 6 h after acute lung injury (ALI) induced by Escherichia coli lipopolysaccharide (LPS) avoided the pulmonary lesions (Rocco et al., 2003). Thus, we administered LASSBio 596 6 h after the exposure to MCYST-LR and the experiments were carried out 2 h afterwards.

Microcystin administration triggers a network of inflammatory responses mediated by immune cells in many organs (Wang et al., 2008). It inhibits PP1 and 2A, yielding an unusual cellular protein phosphorylation, and, thus, possibly activating protein kinase C. The latter activates phospholipase A2 and cyclooxygenase, triggering inflammation (Nobre et al., 2001; Kujbida et al., 2008).
The influx of PMN also yields the production of reactive oxygen species (ROS), generating oxidative stress that adds to the development of tissue injury (Moreno et al., 2005).

So far the handling of lesions induced by MCYST-LR has been mostly prophylactic (Nobre et al., 2001; Dawson, 1998). No work was found in the literature concerning the treatment of acute lung inflammation induced by MCYST-LR.

LASSBio 596, a novel anti-inflammatory drug candidate, structurally designed as a hybrid of thalidomide and sildenafil, exhibits important anti-inflammatory and immunomodulatory profiles by inhibiting PDE 4 and 5, which regulate the breakdown of the intracellular second messengers cAMP and cGMP, respectively, and by modulating the TNF-α levels (Lima et al., 2002; Rocco et al., 2003). The structure of this new achiral thalidomide hybrid lacks the phthalimide and glutarimid rings Fig. 3. Photomicrographs of lung parenchyma stained with hematoxylin–eosin. CTRL, TOX, L596, and DEXA, animals that received saline, microcystin-LR, microcystin-LR plus LASSBio 596, and microcystin-LR plus dexamethasone, respectively. Original magnification = ×200.

Fig. 4. Alveolar collapse and influx of polymorphonuclear (PMN) cells. CTRL, TOX, L596, and DEXA, animals that received saline, microcystin-LR, microcystin-LR plus LASSBio 596, and microcystin-LR plus dexamethasone, respectively. Bars are mean ± SEM of 8–10 animals in each group. Data were gathered from ten random, non-coincident fields per mouse. Different letters indicate significantly different values (p < 0.05).
(responsible for the teratogenic effects of thalidomide), thus possibly avoiding an eventual unwanted effect (Lima et al., 2002). The precise mechanism whereby LASSBio 596 attenuates lung inflammation is not known. According to the literature PDE 4 and 5 inhibitors may lead to the suppression of chemoattractant and pro-inflammatory cytokine release, as TNF-α and IL-1β (Turner, 1993; Miotla et al., 1998), the downregulation of cell adhesion molecules, the inhibition of leukocyte migration, functional inhibition of various types of cells including lung macrophages, neutrophils, lymphocytes, and monocytes, and increased macrophage anti-inflammatory cytokine production (Miotla et al., 1998). LASSBio 596 modulates lung inflammation and blocks fibroproliferation in LPS-induced ALI (Rocco et al., 2003). In addition LASSBio 596 effectively prevents lung mechanical, morphometrical changes and blocks fibroproliferation in a murine model of chronic asthma (Campos et al., 2006). Thus, we tried this compound to treat inflammatory reactions to MCYST-LR.

Respiratory mechanical parameters were measured by the end-inflation occlusion method. This method allows the identification of elastic, resistive, and viscoelastic and/or inhomogeneous lung mechanical components (Bates et al., 1985, 1988). TOX data were similar to those previously reported (Soares et al., 2007). L596 inhibited alterations in all mechanical parameters, while DEXA did not avoid the increase in resistive pressure (that reflects airway resistance) and total pressure (Figs. 1 and 2). Interestingly, dexamethasone has broad clinical applications, including pulmonary diseases; however, in our model the drug was not able to avoid lung dysfunction induced by MCYST-LR. This finding can be explained by histopathological analyses that show discreet edema, thickening of the alveolar septa due to neutrophil recruitment and an increase in collapsed areas in all groups that received MCYST-LR. Additionally, L596 and DEXA prevented alveolar collapse, the former to a greater extent than the latter, suggesting that both therapies might prevent lung functional alterations caused by MCYST-LR (Figs. 3 and 4).

Free MCYST-LR was not present in mice lungs, but was detected in their livers. The amounts were similar in all groups that received MCYST-LR. The liver is the target organ for microcystins, because of the ability of hepatocytes to uptake these toxins through bile acid transporters and organic anion transporting polypeptides (Carmichael, 1994; Feurstein et al., 2009). Moreover, i.p. injection favors microcystin transportation straight to the liver, as the toxin initially reaches the portal circulation, and, then, the liver. Indeed, 70% of the microcystin reaches the liver 1 min after...
mice intraperitoneal exposure and after 3 h 90% of the toxin were found in the liver (Brooks and Codd, 1987; Robinson et al., 1989, 1991). A damaged liver can release inflammatory mediators causing a secondary lung inflammation (Nobre et al., 2001). Furthermore, inflammatory mediators (TNF-α and IL-1) can be produced by peritoneal macrophages after microcystins injection (Nakano et al., 1991). Thus, if MCYST-LR did not reach the lungs in our model, probably the acute pulmonary inflammation was started by cytokines produced by the damaged liver and peritoneal macrophages, which were carried by the blood stream. Another possibility is the direct action of MCYST-LR on lung cells. However, a normal activity of PP1 and 2A in lung of mice intraperitoneally injected with microcystin-LR was reported (Soares et al., 2007), which means that both enzymes were not inhibited. In this context, alveolar macrophages stimulated by MCYST-LR can produce prostaglandins F2 and PGE2, as well as tromboxane B2 and arachidonic acid (Naseen et al., 1989). The toxin could also damage type II pneumocytes. Since our method did not allow the determination of bound MCYST, it was not possible to confirm its presence in the lung.

We found a hepatic pathological profile similar to those previously described (Hooser et al., 1989; Fugiki, 1992; Carmichael, 1994; Barreto et al., 1996; Azevedo et al., 2002; Andrinolo et al., 2008). In our study no treatment was effective in reversing the hepatic toxic lesion of MCYST-LR (Fig. 7). Possibly, the short duration of treatment (2 h), the type of aggression (direct to the liver and indirect to the lungs), and/or the anti-inflammatory molecules used were not able to reverse the effects caused by PP inhibition in liver cells.

SOD is the primary class of enzymes that initiate the process of detoxifying superoxide anion by converting it into H2O2, which is a substrate for CAT and GSH-Px (glutathione peroxidase) (Moreno et al., 2005). SOD consumption (Fig. 5) indicates that an important production of O2•− occurred in groups TOX and DEXA. CTRL and L596 had SOD activity preserved. It might be postulated that LASSBio 596 probably has a SOD-like activity, i.e., it scavenges upon superoxide anions. Furthermore, thalidomide is able to stabilize neutrophil membrane, reducing the production of ROS, which could take part in the protective effect of LASSBio 596 (Tadesse et al., 2004). Finally, it is known that steroids diminish SOD activity (Zafir and Banu, 2009), which could explain our findings (Fig. 5). The activity of MPO was increased in TOX possibly to detoxify hydrogen peroxide. Since peroxide was not formed as a result of SOD activity in L596, the latter would behave like CTRL, but an increase in MPO level would be expected in DEXA, which was not

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**Fig. 7.** Photomicrographs of liver parenchyma stained with hematoxylin–eosin. CTRL, TOX, L596, and DEXA, animals that received saline, microcystin-LR, microcystin-LR plus LASSBio 596, and microcystin-LR plus dexamethasone, respectively. Original magnification = ×400. *: necrosis; #: binucleated hepatocytes; ellipse: hyalinization; rectangle: cytoplasmatic vacuolization.
verified. However, it cannot be forgotten that steroids stabilize lysosomal membranes, thus avoiding MPO release by PMN (Rugstad, 1988). The same reasoning could be applied to CAT (Zafir and Banu, 2009). As a final result, TOX presented higher TBA levels than CTRL, L596 and DEXA that did not differ among them. Oxidative damage, as indicated by TBARS, was present solely in TOX. In this line, exposure to MCYST-LR increases the products of lipid peroxidation, as evidenced by accumulation of TBARS in liver and kidney of rats and fish (Moreno et al., 2005). Taken together, our results indicated that in this model of lung inflammation oxidative stress was avoided principally by LASSBio 596.

5. Conclusion

The results of present study showed that both LASSBio 596 and dexamethasone reduced the inflammation and changes in lung mechanics induced by MCYST-LR, but the beneficial effects of LASSBio 596 outperformed those of dexamethasone. LASSBio 596 effectively prevented respiratory mechanical changes, minimized lung morphometrical alterations, inflammation and oxidative stress in a mouse model of microcystin-induced acute lung injury. Although mouse and man share many basic physiological processes, the results reported herein cannot be extrapolated directly to humans.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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