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First evidence of estrogenic potential of the cyanobacterial heptotoxins the nodularin-R and the microcystin-LR in cultured mammalian cells

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ABSTRACT

The estrogenic activity of cyanobacterial hepatotoxins microcystin-LR (MC-LR) and nodularin-R (NOD-R) was for the first time investigated *in vitro* in a stably transfect cell line with an estrogen-regulated luciferase gene. Treatment of cells with NOD-R caused a dose-dependent increase in the luciferase activity. NOD-R gave rise to an induction of luciferase activity with an EC_{50} value of 66.4 nM, whereas the positive control, 17β-estradiol (E2) had an EC_{50} of 9.6 pM. This indicates that NOD-R is a 6900-fold weaker inducer of luciferase than E2. In contrast, only a slight but significant activation of the luciferase gene was observed with MC-LR between 2.01 and 60.1 nM, and a maximal-induced response was observed with 10.1 nM, approximately 25% of the maximal effect obtained with 1 nM E2. The decrease in the luciferase activity at high MC-LR concentrations can be explained by a cytotoxic effect. No synergistic estrogenic effect was observed when each toxin was co-administrated with E2. However, the induction of the luciferase activity by NOD-R and MC-LR was inhibited by co-treatment with 1 μM of the pure estrogenic receptor (ER) antagonist ICI 182,780, thus proving the ER-dependency of the estrogenic effect.

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1. Introduction

Cyanobacteria are often associated with hepatotoxic freshwater blooms worldwide. Certain species of these microorganisms are capable of producing a variety of potent toxins, including a group of hepatotoxins known as microcystins and nodularins [1]. These toxins have caused mortality in animals [2] and illness in humans [3] or, when exposed through hemodialysis, even death [4,5]. These health threats have led the World Health Organization (WHO) to establish a provisional guideline value for microcystin-LR of 1 μg/L drinking water [6].

Approximately 70 variants of microcystins have been isolated from cyanobacterial blooms and cultures, with microcystin-LR as the most common [1]. Microcystin-LR (MC-LR) and nodularin-R (NOD-R) are cyclic hepatotoxins with a molecular weight of 994 and 824, respectively (Fig. 1A and B). Their toxicities resulted on the inhibition of catalytic units of serine/threonine protein phosphatases types 1 (PP-1), 2A (PP-2A), and 3 (PP-3) [7]. Therefore, these toxins modulate the expression of oncogenes, early-response genes, and tumour necrosis factor α, and affect cell division, and apoptosis [8–11], and they are considered as power tumour promoters in experimental animals [12,13]. However, the significance of this for humans, who may be subjected to chronic exposure via drinking water, remains unclear. Overall, the evidence for carcinogenecity of these hepatotoxins is considered inadequate today in humans and limited in animals [14]. Recently, MC-LR has been classified as "possibly carcinogenic to humans" (group 2B), and NOD-R as "not classifiable as to their carcinogenicity" (group 3) [15].

In addition, in a long-term chronic study in mice, Falconer et al. [16] exposed animals for one year to microcystins extract in drinking water, and then male and female toxin-exposed mice were mated and the number, viability, and body weight of the young measured. The results of this last study concluded that there are no differences in the number of young or in the weight, viability, or gender of young between chronically exposed females and control females mated with chronically exposed males and control males, respectively. Thus from this study authors concluded that there is no current evidence for endocrine-disrupting effects of microcystins on reproduction in mammals. In a recent study, Li et al. [17] reported that exposure of male rats to 15 μg/kg/day of MC-LR for 28 days led to the decrease of testis weight, sperm concentration, and the levels of serum testosterone, FSH and LH hormones. Nevertheless, damage induced in humans by repeated consumption of weak quantities of microcystins is probably more frequent and remains more worrying than acute intoxication. Indeed, many aspects concerning these toxins remain unknown, notably those related to their endocrine-disrupting effects on reproduction.

During the last decade there was increasing public concern for the contamination of food and water by endocrine-disrupting compounds (EDCs), chemicals that alter the functions of the endocrine

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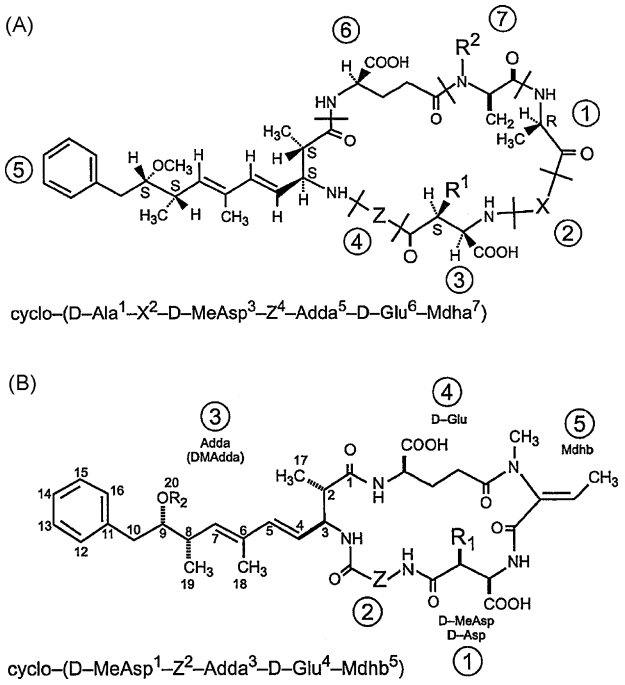

Fig. 1. Chemical structures of (A) microcystin-LR (X = L-leucine (L) and Z = L-arginine (R)) and (B) nodularin-R (Z = L-arginine (R)).

system and cause reproductive disorders [18–21]. A large class of EDCs is called xenoestrogens because they mimic the actions of estrogen hormones naturally produced by an organism [22]. These compounds include both environmental contaminants with binding affinities for the oestrogen or androgen receptors, such as nonylphenol, methoxychlor, phtalates [23–25], as well as human and veterinary pharmaceuticals released into the environment, including ethinylestradiol (EE2) and trenbelone [26]. The hypothesis suggesting that the contribution of environment contaminants towards a suite of disorders in humans, including debate over decreased male fertility due to reduced sperm counts and increased rates of breast cancers is not supported by many research studies [27]. Among these most widely distributed chemicals, natural substances with endocrine activity such as phytoestrogens and mycoestrogens [28,29] and likely hepatotoxins of cyanobacteria have to be taken into account for a holistic evaluation.

Different methods have been used to evaluate the estrogenic activity of xenoestrogens, among them a number of *in vitro* assays including the competitive estrogen receptors (ERs) binding assay [30], the proliferation assay (E-screen) in the human breast cell line MCF-7 [31], the vitellogenin induction assay in primary hepatocytes taken from fishes [32], and the receptor–reporter gene assays using transgenic human cell lines [33] or yeasts [34], which are faster and more cost-effective screening methods for estrogenicity of xenobiotics.

The aim of the present study is to determine the estrogenic and anti-estrogenic potentials of microcystin-LR (MC-LR) and nodularin-R (NOD-R) *in vitro* in a stably transfected cell line (MELN) with an estrogen-regulated luciferase gene by measuring the effects of these cyanotoxins on the induction of the luciferase activity.

2. Materials and methods

2.1. Chemicals

Both cell culture products and fetal bovine serum (FBS) were purchased from Life Technology (Cergy-Pontoise, France). Nodularin-R (CAS No.: 118399-22-7) and microcystin-LR (CAS No.: 101043-37-2) were purchased from Calbiochem-Novabiochem Co. (France). The ICI 182,780 (estrogen receptor antagonist) was kindly provided by Dr. J.M. Renoir (UMR CNRS 8612, University Paris-Sud 11). Dimethylsulfoxide (DMSO), 17β-estradiol (CAS No.: 50-28-2), and all other chemicals were purchased from Sigma–Aldrich Chemical (Saint Quentin Fallavier, France). The test chemicals (NOD-R, MC-LR, E2, and ICI 182,780) were dissolved in DMSO, and the concentration of DMSO in the media did not exceed 0.1%.

2.2. Routine cell culture

The MELN cell line derived from the MCF-7 human breast carcinoma cell line in which an estrogen-regulated luciferase gene was stably transfected [35,36] was kindly provided by Dr. P. Balaguer (INSERM U439, Montpellier, France). This MELN bioassay has been widely used for the detection of estrogenic activity in complex environmental samples [35,36]. MELN cells were routinely maintained in 25 cm^2 culture flasks in Dulbecco's modified eagle medium (DMEM) supplemented by 10% FBS, 2 mM L-glutamine and 1% (v/v) of penicillin–streptomycin solution (10,000 U/mL–10,000 μg/mL) in a humidified incubator at 37 °C and 5% CO_2. Cells were subcultured at approximately 70% confluence over a maximum of 10 passages and regularly tested negative for mycoplasma.

2.3. In vitro estrogenicity bioassay: MELN bioassay

The estrogenicity biossay conditions were used according to those previously described [37]. Briefly, the seeding medium in the 25 cm^2 culture flasks containing cells at approximately 70% confluence was aspirated and the attached cells rinsed with 3 mL of phenol-free DMEM. The rinse medium was then quickly removed and replaced with 3 mL of the experimental medium consisting of phenol-free DMEM with Charcoal-Dextran stripped FBS (DC-DMEM), 2 mM L-glutamine, and 1% (v/v) of penicillin–streptomycin solution (10,000 U/mL–10,000 μg/mL). Cells were then incubated in a humidified incubator at 37 °C and 5% CO_2 for 72 h. After incubation, cells were trypsinized and seeded in 96-well plates at initial concentration of 12,000 cells/well in a volume of 200 μL of the experimental medium DC-DMEM. Cells were then allowed to attach for 24 h in a humidified incubator at 37 °C and 5% CO_2. After incubation, the medium was aspired and then replaced with the experimental medium containing the test chemicals diluted extemporaneously in DMSO with concentration never exceeding 0.1% (v/v) in culture medium, concentration that caused no adverse effect on cell viability and luciferase assays. Cells with test chemicals at different concentrations were then incubated for 24 h in a humidified incubator at 37 °C and 5% CO_2. Each plate contained 1 row (3 wells) negative controls (CD-DMEM), 1 row vehicle controls (CD-DMEM + DMSO at final concentration of 0.1%, v/v), 5 rows positive controls (CD-DMEM + E2 at final concentrations of 10^{-13}, 10^{-12}, 10^{-11}, 10^{-10} and 10^{-9} M), 7 rows NOD-R (CD-DMEM + NOD-R at final concentrations of 2.43×10^{-9}, 6.01×10^{-9}, 2.43×10^{-8}, 4.86×10^{-8}, 7.29×10^{-8}, 1.09×10^{-7} and 1.46×10^{-7} M), and 6 rows MCYST-LR (CD-DMEM + MC-LR at final concentrations of 2.01×10^{-9}, 5.03×10^{-9}, 1.01×10^{-8}, 2.01×10^{-8}, 4.02×10^{-8} and 6.04×10^{-8} M). All chemicals were tested in triplicate and experiments repeated three times.

Twenty-four hours after the treatment, the assay was terminated by placing the plates on ice for 1 min before removing the experimental media. Cells were washed twice with 200 μL ice-cold phosphate buffered saline (PBS), and were then lysed for 10 min at 4 °C with 50 μL lysis buffer (luciferase Kit, Roche Applied Science, Meylan, France). After shaking the plate and allowing to stand on ice for 5 min, aliquots (30 μL) from each well was collected and the luciferase activity was measured for 15 s after injection of 30 μl of

D-luciferin reagent (luciferase Kit, Roche Applied Science, Meylan, France) using a Lumat LB 9507 luminometer (Berthold, France). The luciferase activity was calculated in Relative Luminescence Units (RLU), and results of the test chemicals are therefore expressed as relative estrogenic activities of the maximum luciferase activity (100%) for E2 at 1 nM. The EC_{50}, representing the dose at which the response is 50% of the maximal response for E2 (1 nM), was then calculated for each tested compound. Relative estrogenic potencies are expressed as E2 equivalency factors (EEFs) and are calculated by dividing the EC_{50} for E2 by the EC_{50} for each test compound.

2.4. Anti-estrogenic activity in the MELN bioassay

Anti-estrogenic activity of NOD-R and MC-LR was assessed in the *in vitro* assay (MELN bioassay) using the same procedure used in the estrogenicity assay. Cells were co-administered with 10^{-12} or 10^{-9} M of E2, concentrations that induce significantly minimum and maximum estrogenic response, respectively, and NOD-R at 72.9 nM ($\approx EC_{50}$) or MC-LR at 10.1 nM (concentration inducing maximum estrogenic response). In parallel, concentration of 1 μM of the known competitive estrogen receptor antagonist ICI 182,780 was included as positive control in the anti-estrogenicity experiments, and to verify particularly that the reporter gene activity was strictly estrogen receptor (ER) mediated. All chemicals were tested in triplicate and experiments repeated three times.

2.5. Cell cytotoxicity assay

Cell viability was assessed using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay [38]. Cells were seeded and treated using the same nominal concentrations and procedures used in the estrogenicity bioassay. Twenty-four hours after treatment with test chemicals, or DMSO as a vehicle control, medium was removed and replaced with 200 μL of 0.5 mg/mL MTT solution in sterile PBS. Cells are then incubated for 3 h in a humidified incubator at 37 °C and 5% CO_2. After the incubation period, the formed formazan crystals were solubilized by adding 100 μL of DMSO in each well. After shaking the plate for 15 min at room temperature, the spectrophotometrical absorbance of the formazan product was measured using a microtiter plate reader (Bio-Tek Instruments, ELX800G). Absorbance was measured at 540 and 630 nm (reference value then subtracted from that measured at 540 nm), and the percentage of cell viability was calculated relative to the DMSO as vehicle control. All chemicals were tested in triplicate and experiments repeated three times.

2.6. Statistical methods

Results are presented as mean ± standard error of the mean (SEM). Data were compared for significant differences using Statistica program (version 5) and Student's *t*-test. The levels of significance chosen were $p < 0.05$ and $p < 0.01$.

3. Results

3.1. Cytotoxicity of E2, NOD-R and MC-LR

Concurrent to luciferase activity induction, the effects of E2, NOD-R, and MC-LR on cell viability were investigated by measuring the MTT activity. With the exception of MC-LR, none of the E2 (10^{-13} to 10^{-9} M) or NOD-R (2.43×10^{-9} to 1.46×10^{-7} M) compounds at treated level significantly ($p < 0.05$) altered the MTT activity relative to DMSO-dosed MELN cell lines (negative control) as shown in Fig. 2. MC-LR only at high concentrations (40.2 and 60.4 nM) significantly ($p < 0.05$) decreased the MTT activity relative to vehicle control (Fig. 2).

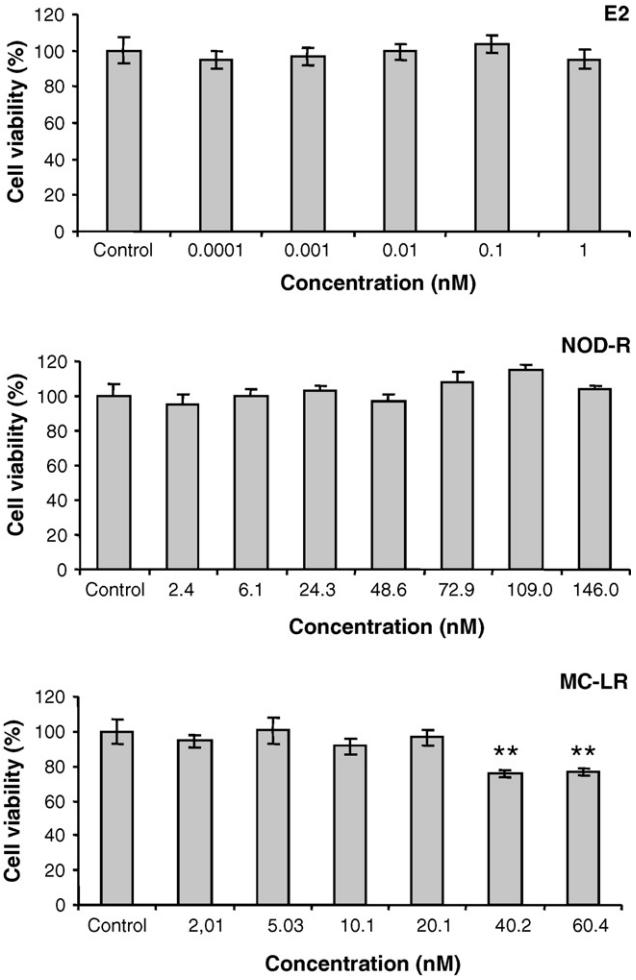

Fig. 2. Viability (%) of MELN cells incubated in the presence of a range of concentrations of E2, NOD-R and MC-LR expressed relative to the DMSO control and assessed by the MTT assay. Values are mean ± SEM from three separate experiments performed in triplicate. $^{**}p < 0.01$ indicates significant difference from the DMSO control.

3.2. Estrogenicity of E2, NOD-R and MC-LR

Sensitivity and reproducibility of the MELN bioassay were assessed by measuring the response to E2. In good agreement with literature values [35], E2 induced activation of the luciferase gene in a concentration dependent manner range from 10^{-13} to 10^{-9} M (Fig. 3). The maximum luciferase activity (100%) was observed with 1 nM E2, and results of the test chemicals (NOD-R and MC-LR) are therefore expressed as relative luciferase activities of this maximum value. The relative luciferase activity (5.9 ± 0.6%) of the vehicle control (DMSO at 0.1% final concentration) was not significantly different from that of the experimental medium. Specificity of this assay was then assessed by determining the induction of luciferase activity with two cyanobacterial hepatotoxins, NOD-R and MC-LR (Fig. 3), and compared their estrogenic potential to E2. E2, NOD-R, and MC-LR, concentration–response curves were then generated in at least three separate experiments (Fig. 3). The pentapeptide hepatotoxin (NOD-R) was significantly effective at inducing luciferase activity in a concentration dependent manner range from 2.43×10^{-9} to 1.46×10^{-7} M (Fig. 3). In contrast, only a slight but significant activation of the luciferase gene was observed

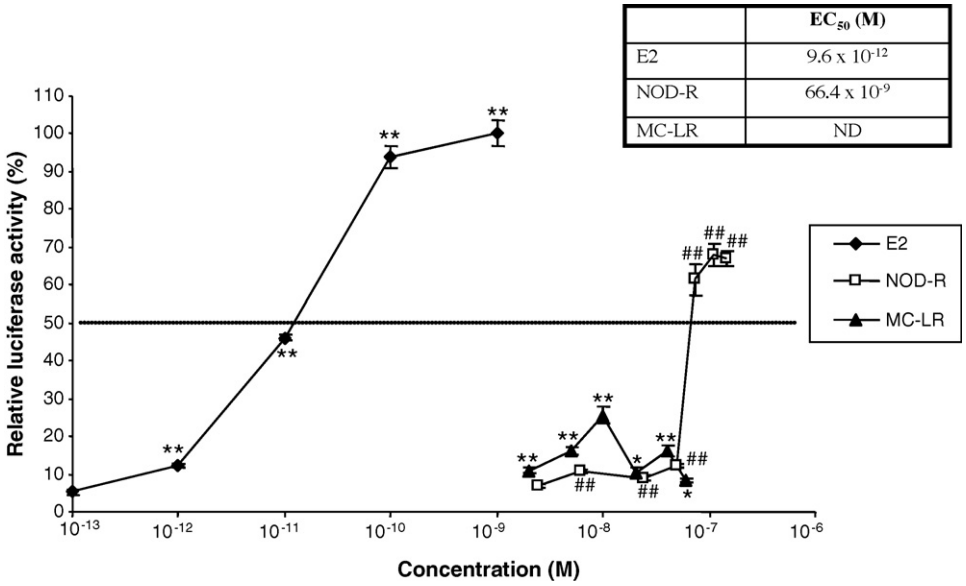

Fig. 3. Dose–response curves of the induction of luciferase activity by E2, NOD-R and MC-LR in the MELN cell lines. Results are expressed as the percentage of the luciferase activity measured per well (mean ± SEM from three separate experiments performed in triplicate). Compound EC_{50} values shown in inset table were extracted from these data and correspond to the point of intersection between horizontal line at 50% relative luciferase activity and dose–response curves on the present figure. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{\#\#}p < 0.01$ indicate significant difference from the DMSO control.

with MC-LR between 2.01×10^{-9} and 6.01×10^{-8} M (Fig. 3). The largest effect was observed with 10.1×10^{-9} M, approximately 25% of the maximal effect obtained with E2 (1 nM). However, at higher concentrations (>10.1×10^{-9} M), MC-LR promoted a decrease in relative luciferase activity (Fig. 3). The estrogenic potential was calculated for each compound by determining their EC_{50} using logarithmic regression in the linear part of the curve. The EC_{50}s obtained for E2 and NOD-R were 9.6×10^{-12} and 66.4×10^{-9} M, respectively (inset table in Fig. 3). Therefore, NOD-R with a relative estrogenic potency of 1.45×10^{-4} was 6900-fold less active than E2.

3.3. Modulation of estrogenic effect of E2 by NOD-R and MC-LR

The possibility that NOD-R and MC-LR might interfere with E2-induced activation of luciferase reporter gene in MELN cells line was also investigated. To this end, MELN cells were incubated with fixed concentrations of NOD-R (72.9 nM) and MC-LR

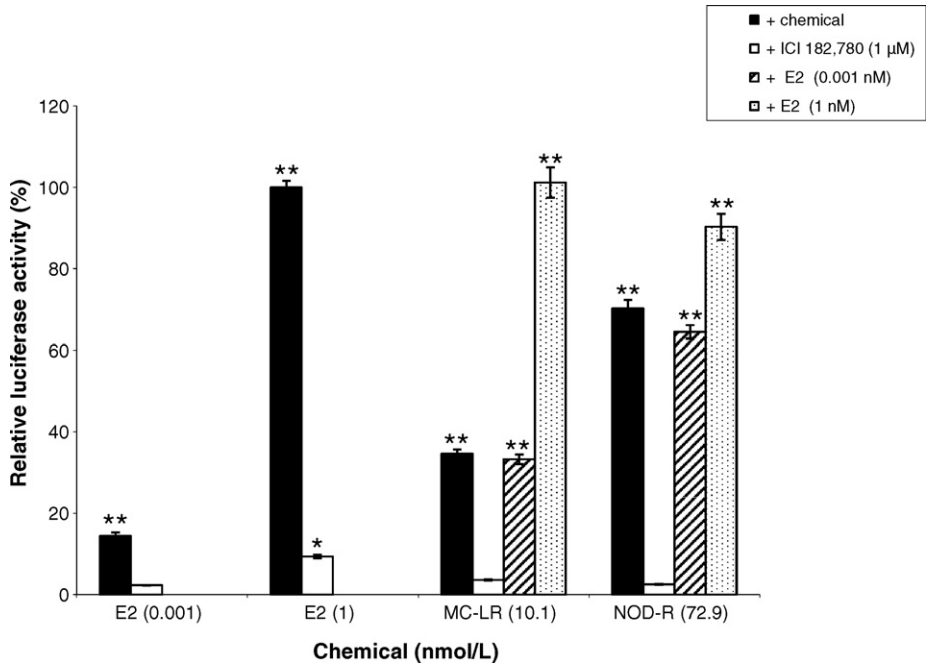

Fig. 4. Relative luciferase activity (%) in the MELN cell lines induced by 72.9 nM of NOD-R or 10.1 nM of MC-LR alone ("compound alone"), in the presence of two concentrations of ER-agonist ("+E2 min" or "+E2 max") or in the presence of 1 μM ER-antagonist ("+ ICI 182,780"). "E2 min" and "E2 max" correspond to E2 at 0.001 and 1 nM, concentrations inducing the smallest and highest luciferase activity significantly different from control, respectively. The E2 was also incubated alone or in the presence of 1 μM ICI 182,780 as positive control. Results are expressed as the percentage of the maximum luciferase activity value obtained for 1 nM E2 (100%) measured per well (mean ± SEM from three separate experiments performed in triplicate). Significant difference between the DMSO control and chemical treatments for each dose are represented by $^{*}p < 0.05$, and $^{**}p < 0.01$.

(10.1 nM) and combined with 0.001 or 1 nM of E2, concentrations that induced significantly minimum and maximum estrogenic response, respectively (Fig. 3). As shown in Fig. 4, MC-LR (10.1 nM) when co-administrated with 0.001 or 1 nM of E2, promoted increase of the relative luciferase activity to equivalent levels compared to those obtained with MC-LR alone (Fig. 4). Similar results were also observed with NOD-R, although levels obtained with NOD-R + E2 were significantly slightly lower than that with NOD-R alone (Fig. 4). Therefore, both NOD-R and MC-LR did not act synergistically with E2 in the induction of the luciferase activity, although NOD-R presented a weaker anti-estrogenic effect.

In parallel, E2, NOD-R, and MC-LR were tested individually with the pure oestrogen receptor antagonist ICI 182,780 (1 μM) in order to determine whether the induction of luciferase activity on MELN cells was ER-mediated. NOD-R (72.9 nM), MC-LR (10.1 nM), or E2 (0.001 and 1 nM) were added to cell cultures together with ICI 182,780 at 1 μM and the relative luciferase activity of substances alone and plus ICI 182,780 were compared (Fig. 4). When each of the test compounds (E2, NOD-R, and MC-LR) and ICI 182,780 were added together, a total antagonism was seen (Fig. 4).

4. Discussion

Many cytotoxic mechanisms concerning MC-LR and NOD-R are relatively insufficiently known, notably those related to their endocrine-disrupting effects on reproduction. Nevertheless, strong evidence supported a plausible tumour promoter mechanism for these liver toxins. This mechanism is mediated via the inhibition of serine/threonine protein phosphatases 1, 2A, and 3 [7,8]. Because the importance of phosphorylation in the activation of the oestrogen receptors has been documented (see in [39]), we suggest that the cyanobacterial hepatotoxins NOD-R and MC-LR could have an estrogenic potential. In our knowledge, this is the first report on the estrogenic potential of MC-LR and NOD-R *in vitro* in a luciferase-based reporter gene system with MELN cells at concentrations lower than those found in natural environments [1,40]. The dose–response curve recorded for the positive control E2 (Fig. 3) was in agreement with previously published data [35], indicating that the assay was functioning properly in our hands. In the absence of cytotoxicity (Fig. 2), E2 and NOD-R concentration dependently induced luciferase activity already at picomolar and nanomolar concentrations, respectively (Fig. 3). At higher concentrations the effect with NOD-R reached a plateau, luciferase activity being maximally increased to 70% of the maximal effect (100%) obtained with E2 (1 nM). The observed EC_{50}-values for E2 and NOD-R were 9.6 pM and 66.4 nM, respectively. Therefore, NOD-R with a relative estrogenic potency (EEF) of 1.45×10^{-4} was 6900-fold less active than E2. Although NOD-R exhibited an approximately 6900-fold weaker estrogenic potential than the endogenous hormone E2, its estrogenicity is higher than those of the environmental estrogens bisphenol A (EEF = 2.5×10^{-5}), and 4-nonylphenol (EEF = 1.25×10^{-5}) obtained using luciferase reporter gene assays with MVLN cells [41]. In contrast, though MC-LR-exposure induced also the luciferase activity, the level enhancement was significant ($p < 0.001$) and reached maximum value at 10.1 nM concentration before depleting at higher concentrations but remains however significantly higher than controls (Fig. 3). The decrease in the induction of the luciferase activity at high test concentrations of MC-LR can be explained by the cytotoxic effect observed using the MTT assay (Fig. 2). Using MCF-7 (human breast adenocarcinoma) cells, Botha et al. [42] detected a decrease in the cell viability (≤50%) by MTT assays at 50 μM of MC-LR at 24 h treatment. Our results from the MTT assay showed that the viability (≤80%) of the transgenic human cell line MELN (derived from the MCF-7 cell line) was significantly affected by MC-LR at doses (≥40 nM) similar to those previously reported to induce cytotoxicity in primary hepatocytes [43]; however, 500–1000 times lower to those reported for other mammalian cell lines [42,44,45]. These results suggest that MELN cell line may constitute a cell model to evaluate the cytotoxicity of microcystins. However, in the case of low concentrations (<10 nM), in which no cytotoxicity was observed, the induction of the luciferase activity was significantly higher with MC-LR than NOD-R (Fig. 3). This indicates that the dose–response effect of MC-LR on luciferase activity appear more rapidly than NOD-R.

Initially, E2, NOD-R, and MC-LR were evaluated individually in order to establish individual chemical concentration–response data. These data were used later to facilitate the selection of chemical concentrations for use in the mixture studies. Significant responses in the assay system were observed for E2 at 0.001 nM, NOD-R at 72.9 nM, and MC-LR at 10.1 nM, as measured in terms of percentage response relative to 1 nM of the native hormone E2 treatment (Fig. 4). However, a combination of any two of these toxins with E2 at 0.001 or 1 nM did not produced a synergistic increase in luciferase activity as compared with the individual toxin (Fig. 4). Nevertheless, when NOD-R was co-administrated with E2 at 0.001 or 1 nM the induction of the luciferase activity for the binary mixtures was slightly lower then that observed for the NOD-R alone (Fig. 4), indicating a weak anti-estrogenic effect. Moreover, the induction of the luciferase activity by NOD-R and MC-LR was inhibited by co-treatment with 1 μM of the ER antagonist ICI 182,780, thus proving the ER-dependency of the estrogenic effect. Environmental compounds known as xenoestrogens have wide structural diversity, but all have lipophillic phenolic rings and other hydrophobic components in common, a characteristic they share with steroid hormones and related nuclear receptor-activating compounds. In contrast, NOD-R and MC-LR are cyclic peptides with chemical structures non similar to certain natural and synthetic oestrogen-mimicking substances such as genistein, coumestrol, bisphenol A, and nonylphenol, thus they are likely to affect estrogenic responses via some nongenomic signalling pathways. Indeed, recent reports have documented the activation of steroid hormone receptors by ligand-independent pathways as well as synergistic activation of steroid hormone receptors by hormone and protein kinase activators (see in [39]). In fact, all of the steps in transcriptional activation of ER dependent genes, i.e., ligand binding, ER dimerization, DNA binding, and the interaction with cofactors, appear to be influenced by phosphorylation of ERs (see in [39]). For example, Power et al. [46] showed that the okadaic acid, a marine phycotoxin presenting the same mode of action as the hepatotoxins of cyanobacteria type microcystin, activates at a similar concentration (50 nM) than MC-LR and NOD-R estrogen receptors in the absence of their ligands. Because of the similarity of the mechanisms of action of the okadaic acid and microcystins as inhibitors of some serine/threonine protein phosphatases, and the importance of phosphorylations in the activation of the estrogen receptors, we suggest that the estrogenic potentials of NOD-R and MC-LR observed in this study are more likely mediated by signalling phosphorylation pathways. The difference observed in the estrogenic potential between NOD-LR and MC-LR is likely due to the difference in the activity of serine/threonine protein phosphatases revealed in literature between these toxins in spite of their close resemblance in terms of structure and toxicity. In fact, MC-LR inhibits PP2A and PP3 more potently than PP1 (order of potency PP2A ≫ PP3 > PP1); however, NOD-R inhibits PP3 and PP1 at a similar concentration that is 40–80 times higher than that which affects PP2A (order of potency PP2A ≫ PP3 ≈ PP1) [47]. On the other hand, recent studies reported that MC-LR and NOD-R induced oxidative stress [43]. Therefore, oxidative stress could mediate the estrogenic potential of these cyanotoxins by the formation of reactive oxygen species (ROS) which can cause a drastic modulation of oxidized/reduced ration of signalling proteins, such as transcription factors (see in [48]).

In conclusion, the activation of the luciferase gene in the receptor–reporter gene assay using transgenic human cells line MELN indicates clearly that MC-LR and NOD-R at low concentrations present estrogenic potential likely by indirect interaction with estrogen receptors. Therefore, they could act as endocrine disruptor at concentrations lower than those found in natural environments, providing some evidence of their reproductive toxicity.

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