Forensic Relevance of Microcystin Detection: A Comparative Analysis of Cross-Reactivity Factors, Toxicity Equivalency Factors and Inhibition Equivalency Factors

Master's Final Project

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TABLE OF CONTENTS

ABSTRACT

Microcystins (MCs) and nodularin are potent cyanotoxins with significant environmental and public health risks. Accurate and reliable detection and the understanding of their toxicity are crucial for effective risk evaluation and management. This work experimentally investigates the cross-reactivity factors (CRFs) with a colorimetric immunoassay using magnetic beads as supports for detection of MCs. It also reviews and compares the toxicity equivalency factors (TEFs), the inhibition equivalency factors (IEFs) and the CRFs from existing literature.

The optimised conditions for the immunoassay include 10 µg/mL MC-LR for magnetic bead conjugation and a 1:2500 monoclonal antibody (mAb) dilution, ensuring high sensitivity and reproducibility. The assessment of antibody affinity revealed different cross-reactivity among MC variants. Some variants, like MC-RR, MC-YR, MC-WR and nodularin, that share structural similarities, exhibited higher immunoreactivity compared to MC-LY and MC-LW, which did not show effective antibody recognition.

Finally, a comparative analysis of CRFs, TEFs, and IEFs revealed some relationships among these factors. The variability is attributed to diverse experimental parameters, including antibody type, assay format, detection technique, and biological method. Discrepancies between phosphatase inhibition and toxicity suggest an important role of pharmacokinetics. Furthermore, this work identifies potential false negatives (high toxicity and low detection) and false positives (low toxicity and high detection) comparing immunoassay affinity and toxicity. This work underscores the complexity of MC assessment, emphasizing the importance of selecting analytical tools according to the specific purpose and application.

Keywords: Microcystins, nodularin, cyanotoxins, immunoassay, magnetic beads, cross-reactivity factors, toxicity equivalency factors, inhibition equivalency factors

1. INTRODUCTION

1.1. Microcystins and nodularins

The growing global concern about water quality has highlighted the importance of monitoring the presence of emerging contaminants, particularly those that pose a significant risk to human health and the integrity of aquatic ecosystems. Among these contaminants, microcystins (MCs), a group of potent toxins produced by cyanobacteria, have gained particular attention due to their widespread presence and harmful effects [1].

A specific combination of environmental factors, including high nutrient levels, warm water temperatures, and variations in salinity triggers the excessive growth of cyanobacteria [2]. These blooms not only disrupt the ecological balance of the aquatic system but also present serious challenges to water supply safety [3].

Cyanotoxins are structurally diverse chemical compounds with a wide range of toxicological effects that negatively impact aquatic ecosystems, plant life, and, alarmingly, animal and human health. Hundreds of cyanotoxins have been identified and classified according to their target organ into hepatotoxins, neurotoxins, and dermatotoxins. Among them, the hepatotoxic MCs represent the most frequently detected class in aquatic ecosystems and drinking water sources, making them a significant problem to animal and human health [4].

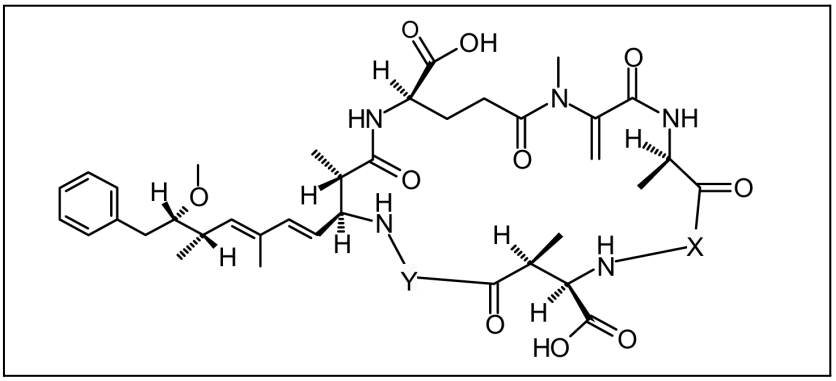
Structurally, MCs are cyclic heptapeptides characterized by the presence of a unique β-amino acid called Adda (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-deca-4,6-dienoic acid), which is exclusive to MCs and nodularins [5]. Nodularin is a pentapeptide structurally similar to microcystins (MCs), and it also exhibits potent hepatotoxicity [6]. Although less frequently encountered than MCs, its potential toxicity also makes it a relevant contaminant in water quality monitoring [7].

The structural variability of MCs results mainly from differences in the amino acids, particularly at positions 2 and 4 of the peptide ring (Fig. 1). For example, MC-LR contains leucine (L) at position 2 and arginine (R) at position 4, while MC-RR contains arginine at both positions [8,9]. Over 80 MC variants have been identified, each exhibiting varying levels of toxicity [6].

To address this variability, Toxicity Equivalency Factors (TEFs) have been developed. When a sample is analysed with instrumental analysis methods, which are based on structural identification of MCs, TEFs provide a way to estimate the overall toxicity of that sample. This is done by comparing the toxic potency of each MC variant to that of the reference compound, MC-LR, which is assigned a TEF of 1. A variant with a TEF of 0.5 would be considered half as toxic as MC-LR. This approach allows the researchers

and regulatory bodies to assess cumulative toxicity, even when multiple variants are present in a single sample.

A)

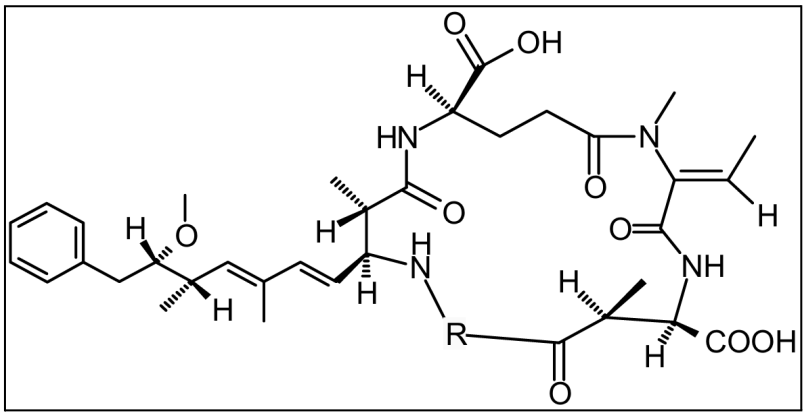


Microcystin

	X	**Y**
MC-LR	Leucine	Arginine
MC-RR	Arginine	Arginine
MC-YR	Tyrosine	Arginine
MC-WR	Tryptophan	Arginine

	X	**Y**
MC-LW	Leucine	Tryptophan
MC-LY	Leucine	Tyrosine
MC-LA	Leucine	Alanine
MC-LL	Leucine	Leucine
MC-LF	Leucine	Phenylalanine

B)



Nodularin

Figure 1. A) General structure of MC (on top) and a table of amino acids in the different MC variants at positions 2 (X) and 4 (Y) (below); B) General structure of nodularin.

MCs act as potent inhibitors of serine/threonine protein phosphatases PP2A and PP1, mechanisms that can induce structural liver damage even at low concentrations [5]. Although the primary target is the liver, MC-LR can also affect the kidney and the gastrointestinal tract [4]. In the context of enzymatic assays, such as Protein Phosphatase Inhibition Assays (PPIAs), Inhibition Equivalency Factors (IEFs) are values that quantify the relative inhibitory potency of different MC variants compared to MC-LR. They are defined as the ratio of the concentration of MC-LR to the

concentration of another MC variant required to achieve 50% of enzymatic inhibition (IC_{50}) under defined conditions.

As mentioned, monitoring MCs is essential due to their global distribution and adaptability to various climates [1,3,7]. Although they are particularly prevalent in temperate and tropical regions, where higher temperatures promote cyanobacterial blooms, climate change and eutrophication have led to an increase in cyanobacterial blooms worldwide. In Catalonia and across the Iberian Peninsula, as in other European countries, several reservoirs have reported blooms during the summer months [4].

Cyanobacterial blooms, and thus MCs, are mainly found in stagnant or slow-flowing freshwater, such as lakes, reservoirs, ponds, and rivers. When favorable conditions occur, such as high temperatures or the presence of nutrients, this type of water is prone to cyanobacterial blooms. Moreover, they can appear in artificial systems, including drinking water treatment plants, swimming pools, or irrigation systems [4]. These toxins can resist conventional filtration processes and may be distributed to agricultural fields, where they may accumulate in crops. This generates a potential risk for animals and humans who consume them [10]. Additionally, MCs have been detected in water from aquaculture facilities, where they can contaminate fish that are later consumed by humans [11].

One of the characteristics of MCs is their bioaccumulation potential in aquatic organisms and plants. This results in indirect human exposure, through the food chain, especially in areas with high agricultural or urban activity that are particularly vulnerable [11]. Consequently, reservoirs used for drinking water are frequently monitored [3].

In response to MCs problematic, the World Health Organization (WHO) has established a guideline value of 1 µg/L for MC-LR in drinking water [11], reinforcing the need for sensitive and reliable methods of detection to protect human health and monitor toxins presence in the aquatic environments [3,12,13].

1.2. Why is it important to detect them?

The detection of MCs is essential for public health and environmental protection. Contaminated water ingestion produces a variety of acute symptoms such as nausea, vomiting, diarrhea, abdominal pain, headache, and fever. Some studies with animals and cellular models demonstrated that MCs can cause gastrointestinal damage,altering the permeability of the intestinal barrier, inducing oxidative stress and cellular damage in the intestine, triggering inflammatory responses and affecting intestinal microbiota [14]. Furthermore, long-term exposure to low concentrations has been linked to potential carcinogenic effects, particularly liver cancer. Other long-term effects on different organ systems are currently under investigation [4,15]. Beyond human health,

MC are toxic to a broad range of animals, including livestock, pets, wildlife, and aquatic organisms that become intoxicated by ingesting contaminated water [5].

Exposure to MCs can occur through: the ingestion of contaminated drinking water, direct contact with contaminated water during recreational activities, consumption of contaminated food that has accumulated toxins (e.g. fish or shellfish), and contaminated Blue-Green Algae (BGA) supplements [11].

Notably, there have been documented different cases of MC exposure in the literature, which are analyzed below. French et al. [16] described three pediatric cases of potential cyanotoxin exposure during the summer/autumn, when harmful algal bloom (HAB) appears in Northwest Ohio. Two children developed persistent nausea, vomiting and acute kidney injury after playing on a beach with a HAB advisory. While gastrointestinal panel tests were negative for common pathogens, no cyanotoxin testing was performed on the patients or the water. The third case involved a child exposed in a wading pool filled with water directly from Lake Erie (also under a HAB advisory), presenting similar gastrointestinal symptoms and elevated liver enzymes, indicative of MC-induced damage. Broadening this perspective, the review by Svirčev et al. [17] consolidates information on historical human MC intoxication events. It prominently features the tragic Caruaru, Brazil incident in 1996, where hemodialysis patients died from acute MC poisoning in contaminated dialysis water, a pivotal event that underscored MCs as a direct and lethal threat. This review also references epidemiological studies in China, particularly in the Lake Taihu region, linking MC exposure in drinking water to increased liver cancer incidence. Further expanding on global incidents, Wood et al. [18] also discusses the Caruaru outbreak and Chinese studies, while additionally highlighting other reported events. These include gastroenteritis outbreaks associated with cyanobacterial exposure in recreational waters like Darling River in Australia and various sites in the United States, as well as skin irritation outbreaks following contact with blooms in the United Kingdom [18]. Wood et al. [18] also details significant acute animal intoxication events, with numerous deaths reported in dogs after swimming or drinking from bloom-affected waters, and large-scale livestock mortalities from consuming contaminated water. Deaths in wild animals, particularly birds and aquatic species, are also acknowledged [18].

Underscoring the critical need for toxin detection and addressing the issue of underreported cases, Tamale et al. [19] highlighted the presence of MCs in Mozambique's drinking water. Despite detecting significantly elevated MC concentrations, their article points out the absence of monitoring programs, and consequently, a lack of official reports of human or animal intoxications and mortalities. The authors suggest that this absence of registered cases stems from

limited monitoring capabilities and the difficulty for public health professionals to recognize and confirm MC intoxication symptoms.

All authors emphasized the challenge of diagnosing cyanotoxin intoxications without invasive testing.

1.3. Detection methods

Given the high toxicity, environmental persistence and resistance of MCs to conventional water treatment processes, the development and application of reliable detection methods are essential for ensuring water safety and protecting public health. Accurate detection is crucial not only for routine public health monitoring, but it also plays an important role in the context of contamination incidents. The ability to accurately identify and quantify MCs and their variants in various environmental matrices is essential for investigating the sources of contamination, reconstructing the timeline of the event, and potentially assigning responsibility in cases of environmental or public health impact. This capacity for detailed and retrospective analysis is crucial for preventing future incidents and implementing effective corrective measures.

1.3.1. Biological methods

Biological methods for MC detection generally involve the use of mice in bioassays, to measure overall toxicity. The mouse bioassay (MBA) was one of the first approaches applied in the detection of cyanobacterial toxins, offering a direct indication of toxic effects. Typically, it involves intraperitoneal injection of extracts containing toxins into mice and observation of physiological responses such as death to estimate toxicity [64].

This method can detect the presence of toxic compounds in general, and therefore it may be non-specific and not selective for MCs [7,20]. Moreover the MBA requires a significant number of laboratory animals and is now considered ethically unacceptable due to animal welfare concerns. In addition, it lacks sensitivity and reproducibility, often producing variable results influenced by the physiological condition of the test animals [7]. As a result, this method has been largely replaced by alternative approaches, such as in vitro enzymatic assays and immunochemical techniques, which offer higher sensitivity and specificity. Nonetheless, in some regions, the MBA may still be employed when no other infrastructure is available. Nevertheless, the results obtained from biological methods are useful for analyzing the toxicity of different variants, from which TEFs can be derived. These TEF values serve to elucidate the toxicity of different variants, one of the purposes of this work, together with the comparison with their inhibition potential or their affinity to specific antibodies.

1.3.2. Enzymatic methods

PPIAs offer an alternative to replace the biological methods by wild or recombinant enzymes. This method also detects toxicity in a sample, related to the enzyme

inhibition, but it may not be specific for MCs, as it may also detect other compounds that inhibit the protein phosphatase activity, such as okadaic acid and its derivatives (marine toxins) [7,15,21]. The most widely used enzyme inhibition assays are those targeting protein phosphatase PP2A and PP1, and are commonly used in the monitoring water samples. PPIAs are valued for simplicity, rapid execution and low cost, which make them suitable for preliminary screening.

Different MC variants may produce different levels of response due to variations in their binding affinity and therefore inhibitory potency. In other words, each MC variant may have a different IEF.

1.3.3. Chemical methods

Analytical methods, such as high performance liquid chromatography (HPLC) coupled to mass spectrometry (MS) or ultra-violet (UV) detection, provide accurate characterisation and quantification of individual MC variants that have available standards, and with high sensitivity. However, lack of commercially available standards for some variants is a limitation [7]. Another disadvantage is that they require trained personal, expensive equipment and the analytical process is time-consuming [7,20]. When used to analyse samples with the purpose to protect human and animal health, it is important to note that they do not provide toxicological information (as they are not based on a toxicological effect, but on a structural recognition) In this regard, the TEFs can be used to translate the multi-toxin MC profile of a sample into an estimation of its toxicological potency. Similarly, the IEFs can be used to translate the multi-MC profile of a sample into an estimation of its inhibitory potency.

1.3.4. Immunochemical methods

Immunochemical methods offer a rapid and sensitive alternative for the detection of total MCs, and this will be the focus of our experimental work [7]. ELISA (enzyme-linked immunosorbent assay) is one of the most commonly used methods to detect toxins. The method includes the use of antibodies to recognize their antigen, in this case MCs. Several polyclonal and monoclonal antibodies have been generated using MC-LR, other MC variants or even the ADDA moiety for immunisation, and have high specificity towards MCs and nodularin variants [7]. However, the affinity toward the different MC variants is different depending on the antibody. This different affinity can be quantified by establishing the cross-reactivity factors (CRFs). CRFs express the relative binding efficiency of an antibody to various MC variants compared to a reference toxin (i.e., MC-LR), typically the one used for immunization. A CRF of 1 indicates equal binding affinity, meaning the antibody recognizes the variant as strongly as the reference. Conversely, lower CRFs suggest reduced recognition. The assay format may have influence on the CRFs.

Several ELISA formats have been described. In the direct competitive ELISA (DC-ELISA), the specific antibody is immobilized on a microtiter plate and the toxin competes with an enzyme-labelled MC conjugate for binding to the antibody [11,13]. In contrast, in the indirect competitive ELISA (IC-ELISA), the toxin or a conjugate form is immobilized on the plate and the free toxin in the sample competes with the immobilized one for binding to a free primary antibody, which is subsequently detected with a secondary antibody, which is enzyme-labelled [7]. In these two immunochemical techniques the signal intensity is inversely proportional to the concentration of MCs present in the sample. The signal detected in ELISAs is usually colorimetric. Nevertheless, other detection techniques include chemiluminescence [3], fluorescence [12] and surface plasmon resonance (SPR). The use of SPR as a detection technique transforms the immunoassay into an immunosensor configuration, enabling rapid and robust real-time detection [7].

The principal limitations of immunochemical methods is that they do not give any indication of which variants are present in a mixture of MC variants and it may not distinguish between toxic and non-toxic variants [13]. Nevertheless, a broad recognition can be advantageous for screening purposes, as it allows for the detection of all or most toxins within the same structural family, providing an estimate of the total concentration of MCs.

2. OBJECTIVES

One of the aims of this work is to develop a colorimetric immunoassay using magnetic beads as supports for detection of MCs. The use of magnetic beads as immobilisation supports provides advantages such as higher surface area available for biomolecule immobilisation, improved assay kinetics, more efficient washing steps or lower matrix effects. Thus, MC is immobilised on magnetic beads and a competition is carried out by introducing a known amount of free toxin and an anti-MC-LR antibody. Next, the unbound antibody and toxins are removed. Finally, with the addition of the secondary antibody and its substrate, TMB, different shades of blue can be observed by colorimetry. This immunoassay strategy is summarized schematically and visually in Figure 2.

Using this method, the goal is to construct calibration curves for different MC variants (MC-RR, MC-YR, MC-WR, MC-LY and MC-LW) and nodularin, and to establish their CRFs. To better understand the analytical performance and toxicological relevance of the immunoassay, the CRF values obtained will be compared with toxicity equivalency factors and inhibitory equivalency factors reported in the literature. This comparison will allow for the analysis of the relationships between immunoaffinity, toxicity and protein phosphatase inhibition.

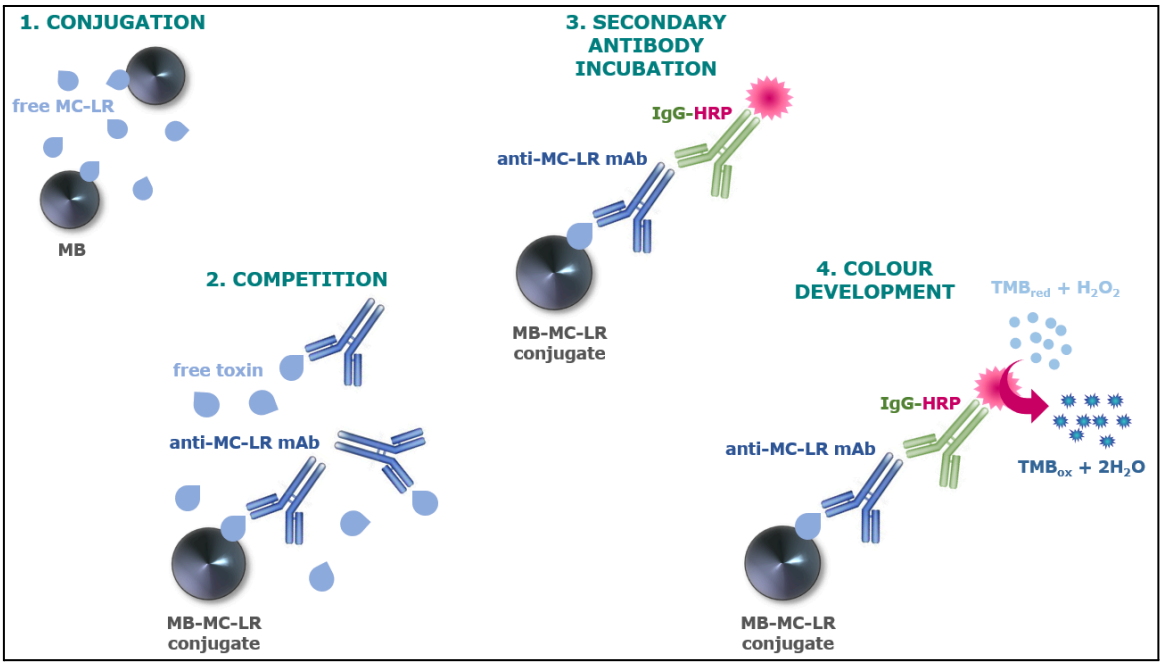


Figure 2. Schematic representation of the key steps of the competitive magnetic bead-based immunoassay for MC detection.

3. MATERIALS AND METHODS

3.1. Reagents, antibody and toxins

MC-LR and the anti-MC-LR mAb (MC10E7) were obtained from Enzo Life Sciences (Lausen, Switzerland). MC-YR was achieved from Laboratorios Cifga (Lugo, Spain). MC-LR and MC-YR standard solutions were prepared in methanol. MC-RR, MC-WR and nodularin were obtained from Palex Medical SA (Sant Cugat del Vallés, Spain). PureCube maleimide-activated MB were obtained from Cube Biotech (Monheim, Germany). Anti-mouse IgG (whole molecule)-horseradish peroxidase antibody (IgG-HRP) produced in rabbit, cysteamine hydrochloride, formaldehyde solution, potassium phosphate dibasic, potassium phosphate monobasic, Tween -20, ethylenediaminetetraacetic (EDTA), bovine serum albumin (BSA) and 3,3',5,5'-tetramethylbenzidine (TMB) were purchased from Sigma-Aldrich (Tres Cantos, Spain).

3.2. Magnetic bead-based immunoassay

The MB-based immunoassay was the following: (1) 10 µL of maleimide-activated MBs were transferred to a 1-mL tube and rinsed three times with 1 mL of washing buffer (0.1 M PBS, 0.05% v/v Tween®−20, pH 7.2) under vigorous mixing; for the washing steps, the tube was placed on the magnetic separation stand and the washing solution was removed; (2) 1 mL of 1 mM cysteamine in binding buffer (0.1 M PBS, 10 mM EDTA, pH 7.2) was added and incubated for 2 h at room temperature; (3) after three washing steps, 1 mL of MC-LR solution (1, 2, 5 or 10 µg/mL for the checkerboard and 10 µg/mL for the final assay) in binding buffer containing 10% v/v formaldehyde was added and

incubated overnight at 4 °C; (4) three washing steps were performed and the MC-LR-coated MBs were resuspended in 1 mL of binding buffer. When amounts of MB varied, volumes were adjusted proportionally. Once the MB-MC-LR conjugate had been prepared, (5) 100 µL of the conjugate was transferred to a 0.5-mL tube, the tube was placed on the magnetic separation stand, the supernatant was removed and 50 µL of the MC-LR, MC variants or nodularin standard solutions and 50 µL of anti-MC-LR mAb at 1/2500, 1/5000 or 1/10.000 dilution for the checkerboard and 1/2500 for the final assay in 2% w/v BSA-binding buffer were added and incubated for 30 min at room temperature; (6) after three washing steps, 100 µL of 1/1000 IgG-HRP dilution in 2% w/v BSA-binding buffer was incubated for 30 min at room temperature; (7) three washing steps were performed and the immunocomplex was resuspended in 100 µL of binding buffer; (8) 95 µL of immunocomplex was transferred to a new 0.5-mL tube and after supernatant removal, 125 µL of TMB liquid substrate was added and incubated for 10 min; (9) the tube was placed on the magnetic separation stand and 100 µL of TMB liquid substrate was collected for colorimetric measurement at 620 nm in a microtiter plate using a microplate reader. All incubation steps were performed under agitation. Experiments were performed in duplicate.

4. RESULTS and DISCUSSION

4.1. Optimization of the magnetic bead-based colorimetric immunoassay

A checkerboard assay was performed to optimize the MC-LR concentration needed for the conjugation of the MBs and the antibody dilution required for the competition assay. Different MC-LR concentrations (10 µg/mL, 5 µg/mL, 2 µg/mL, and 1 µg/mL) were used for the covalent binding to the maleimide-activated MBs through formaldehyde cross-linking to the cysteamine self-assembled on the maleimide groups. At the same time, different antibody dilutions (1:2500, 1:5000, and 1:10000) were tested in the presence and absence of free MC-LR (1 µg/L) in the competition step.

Absorbance values increased with MC-LR concentrations conjugated to MCs and antibody concentrations tested (Fig. 3). Very low absorbance values (around 0.3 and 0.4) were achieved when using 1 and 2 µg/mL of MC-LR conjugated to the MBs. No saturation of the absorbance values at the highest MC-LR concentrations tested was observed, indicating that the MBs were not fully coated at these concentrations. Nevertheless, high enough absorbance values were obtained when using 5 and 10 µg/mL of MC-LR concentrations, indicating that no higher MC-LR concentration was required to perform the assay.

When performing a competitive assay, lower mAb concentrations may provide higher sensitivities, but a compromise between low antibody concentrations and appropriate absorbance values is required. The use of 1:10000 mAb dilution resulted in absorbance values similar to those achieved when no anti-MC-LR antibody was present, indicating

that this dilution was very low for the use in the competitive assay. Absorbance values decreased from 2.1 to 1.1 from 1:2500 to 1:5000 mAb dilution when using 10 µg/mL MC-LR in the conjugation of the MBs (Fig. 3). At the same time, mAb binding decreased from 36% (1:2500 dilution) to 26% (1:5000 dilution), in the presence of 1 µg/L MC-LR in the competition assay, resulting in an increase of the sensitivity of the assay.

$$mAb\ binding\ = \frac{Abs.\ value\ obtained - Abs\ value\ (no\ mAb)}{Abs.\ value\ (no\ MC) - Abs.\ value\ (no\ mAb)}$$

Trends were similar when using 5 µg/mL MC-LR for the conjugation of the MBs, although absorbance values achieved were the half (1.1 at 1:2500 mAb dilution, Fig. 3). Consequently, 10 µg/mL MC-LR for the conjugation of the MBs and 1:2500 mAb dilution in the competition assay were selected to ensure high enough absorbance values and an appropriate difference between the maximum value achieved when no free MC-LR was present and the absorbance value achieved when no mAb was added in the competition. Although with 5 µg/mL MC-LR and 1:5000 dilution of antibody the absorbance values were appropriate, the 10 µg/mL MC-LR and 1:2500 dilution of the antibody were chosen to reduce variability and improve reproducibility.

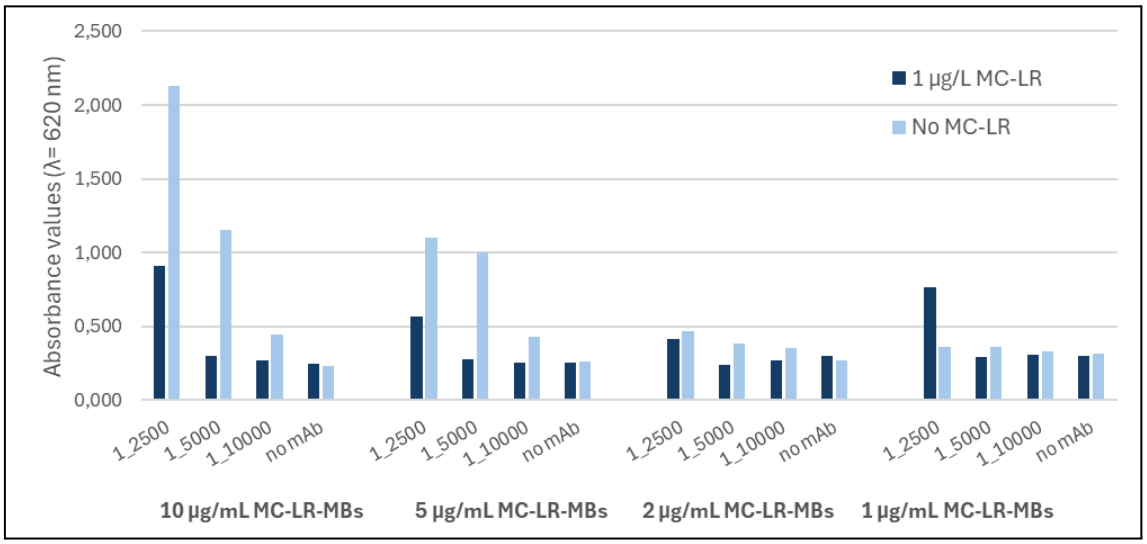


Figure 3. Absorbance values at 620 nm in function of the concentration of MC-LR conjugated to MBs and mAb dilution. Two experimental conditions are shown in different blue: with 1 µg/L of free MC-LR (dark blue bars) and without free MC-LR (light blue bars).

4.2. Assessment of the microcystin variants reactivity with the magnetic bead-based immunoassay

To assess the affinity of the mAb toward different MC variants (MC-LR, MC-RR, MC-YR, MC-WR, MC-LY and MC-LW) and nodularin, two different standard concentrations (100 µg/L and 1 µg/L) were tested in the competition assay and the percentage of antibody binding was calculated. Due to the competitive nature of the assay, mAb binding should be inversely related to the concentration of free MC recognised by the antibody, as fewer antibodies are available to bind the MC-LR conjugated to MB.

For MC-LR, the reference variant, a clear inverse relationship between toxin concentration and antibody binding was observed. At 100 µg/L, absorbance values were low, corresponding to very low binding percentage, close to 0%. In contrast, at lower concentrations (0.1 µg/L and 1 µg/L), the absorbance, and thus the binding percentage increased significantly approaching nearly 100% binding (Fig. 4).

A similar trend was observed with MC-RR, MC-YR, MC-WR and nodularin, although the absorbance values at intermediate concentrations were not analysed. At high toxin concentration (100 µg/L), these variants showed low absorbance values and binding percentages close to 0%, indicating effective competition with MC-LR conjugated to MB for antibody binding. Specifically, average binding at this concentration ranged from 0.9% (MC-WR) to 6.1% (MC-RR), reflecting strong inhibition. At 1 µg/L, all variants showed a marked increase in absorbance, getting close to 100% antibody binding, with average binding percentages between 80.2% (MC-WR) and 99.3% (MC-YR)(Fig. 4). These results suggest that the antibody exhibits relatively high affinity and could recognize these variants, but for the affinity comparison and the calculation of CRF intermediate toxin concentrations are needed.

In contrast to the other variants, MC-LY and MC-LW did not show a typical concentration-dependent inhibition pattern. At the highest tested concentration, 100 µg/L, both variants exhibited high absorbance values, resulting in binding percentages close to 100% (Fig. 4). These results suggest that the free forms of MC-LY and MC-LW are not effectively recognized by the antibody. The elevated binding values indicate antibody affinity <1%.

In summary, the majority of tested MC variants (MC-LR, MC-RR, MC-YR, MC-WR and nodularin) are recognized by the MC10E7 in the competitive MB-based immunoassay. MC-LR, used as the reference variant, showed strong antibody affinity for the antibody. Moreover, variants that share arginine at position 4 (MC-RR, MC-YR and MC-WR) and also nodularin, which have an arginine close to the Adda moiety, and therefore have more similar structure, also exhibit high reactivity. In contrast, the ones that don't share this amino acid in the position 4 (MC-LY and MC-LW) showed low immunoreactivity even at high toxin concentration. These differences underline the variable recognition efficiency of the antibody toward structurally distinct variants and will be further discussed.

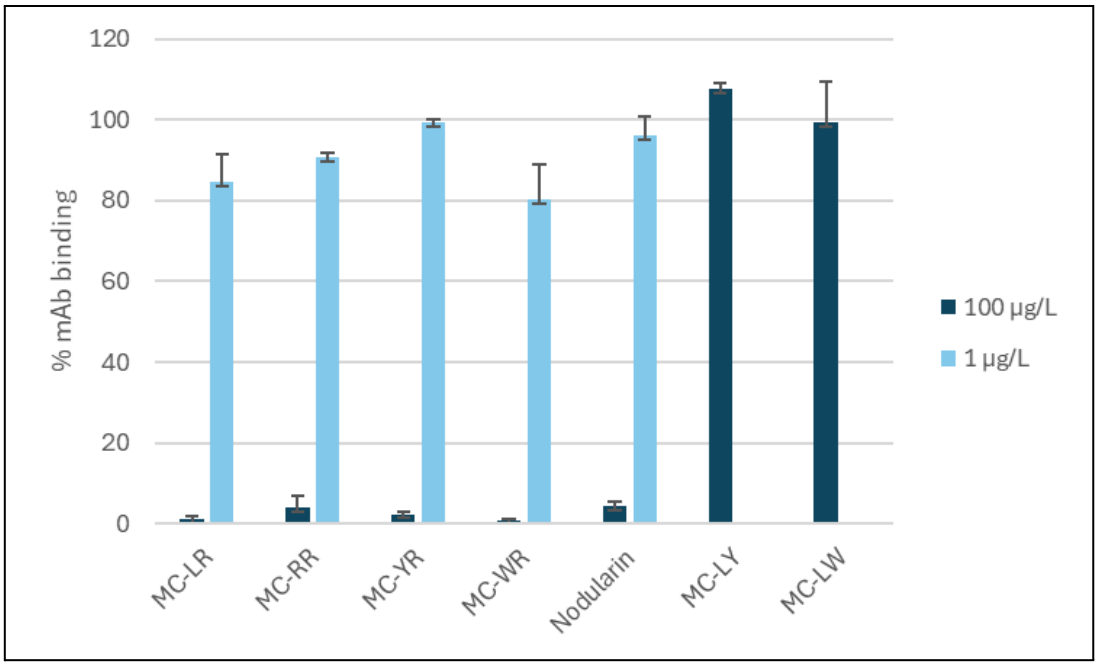


Figure 4. Percentage of mAb binding for different MC variants (MC-LR, MC-RR, MC-YR, MC-WR, MC-LY and MC-LW) and nodularin. MC-LR, MC-RR, MC-YR, MC-WR, and nodularin were tested at two concentrations: 100 µg/L (dark blue bars) and 1 µg/L (light blue bars). MC-LY and MC-LW were tested only at 100 µg/L (dark blue bars). Error bars show SD values (n=2).

4.3. Calibration curves and establishment of the cross-reactivity factors

Calibration curves were generated for each MC variant and nodularin using eight-point serial dilutions, starting from 50 µg/L and diluted 1:2 at each step (50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39). The resulting data allowed for construction of binding curves for each variant, plotted as percentage of binding versus toxin concentration in Figure 5. All curves follow the expected sigmoidal profile of a competitive immunoassay and were fitted to sigmoidal logistic four-parameter equations. The MC-LR curve standing on the left indicates the highest antibody affinity, consistent with lowest IC_{50} value. In contrast, the curve for nodularin and also lightly for MC-YR are displaced to the right, reflecting their lower immunoreactivity (Fig. 5).

These curves were used to determine the IC_{50} for each variant and nodularin. Based on these values, the CRFs were calculated as the ratio of IC_{50} value of MC-LR standard to the IC_{50} value of each variant.

MC-LR, used as the reference compound, exhibited the highest sensitivity, with an IC_{50} value of 1.77 µg/L. This variant serves as the reference point for evaluating the antibody's reactivity to the other variants.

MC-RR, MC-YR and MC-WR displayed moderate cross-reactivity, with IC_{50} values of 2.28 µg/L, 3.44 µg/L and 2.57 µg/L respectively. The CRFs of 0.779, 0.690 and 0.516, respectively, indicate that the antibody exhibits relatively strong recognition of these variants, although lower than that of MC-LR.

In contrast, nodularin showed the lowest cross-reactivity, with an IC_{50} of 4.02 µg/L, corresponding to CRF of 0.441. This suggests a reduced antibody affinity for this toxin, likely due to structural differences (Fig. 1) affecting the epitope recognition.

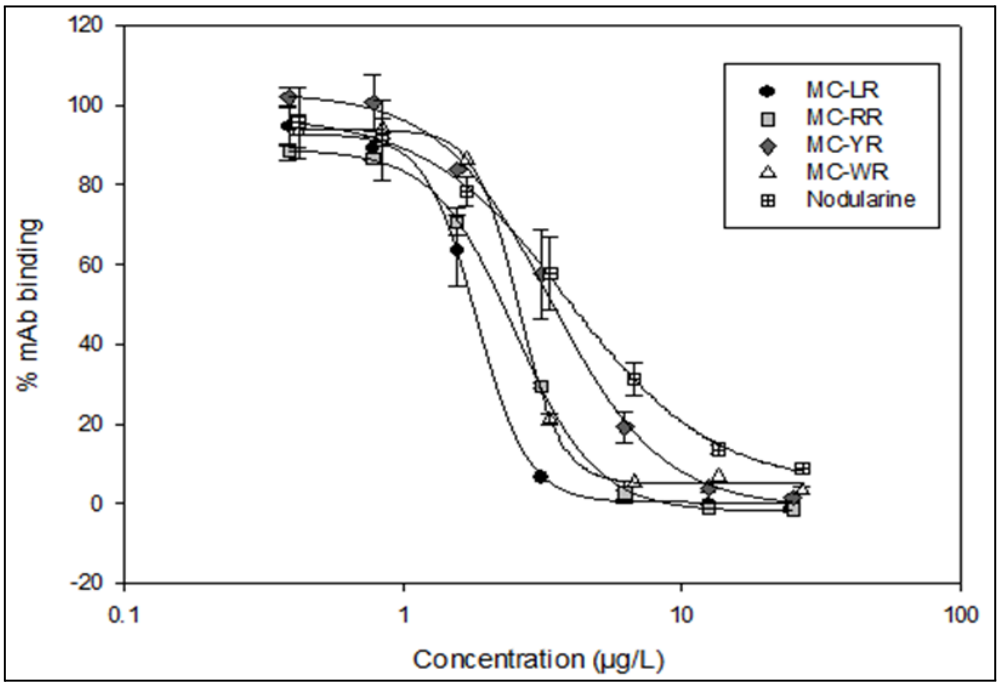


Figure 5. MC-LR, MC-RR, MC-YR, MC-WR and nodularin calibration curves obtained with the MB-based colorimetric immunoassay. mAb binding is expressed as a percentage of the control (no MC). Error bars show SD values (n=2).

4.4. Comparison of cross-reactivity factors

The CRF values compiled in Annex I, derived from this study and an extensive bibliographic review, are important to understanding the specificity of immunoassays for different MC variants and nodularin. These values quantitatively represent how strongly an antibody recognizes each toxin variant relative to a reference standard, which in our case is MC-LR. In Annex I, these values have been classified according to the MC variant. However, due to the use of different formats (e.g., direct competitive ELISA, indirect competitive ELISA, etc.), detection techniques (e.g., colorimetry, fluorescence, etc.) and/or antibodies, direct comparison between studies is complex. Nevertheless, the data allow the identification of general trends in antibody recognition of specific MC variants and nodularin. The CRFs obtained in this study, in which the MC10E7 antibody was used and a colorimetric direct competitive immunoassay was performed, indicate that the antibody shows strong reactivity with MC-RR, MC-YR and MC-WR. It also recognizes nodularin, although with lower intensity. The MC10E7 antibody is considered by many authors to be one of the most sensitive antibodies currently available for MCs detection [4,15]. The MCs recognized by this antibody share an arginine at position 4, an amino acid that is also present in nodularin next to the Adda moiety (the most characteristic region of MCs). These results are in

agreement with previous studies, including those by Zeck et al. [15], Chu et al. [22], and Xu et al. [11], that have described antibodies, which selectively recognize a limited set of MC variants, those containing an arginine residue at position 4 (i.e., MC-LR, MC-RR, MC-YR, and MC-WR).

Discrepancies between CRF values reported in the literature, including those from this study, are evident. These variations can be attributed to several factors, such as the antibody type, its production process and the method used to elucidate the CRF value. Nevertheless, as previously mentioned, some trends are common among studies.

Although not all antibodies perform equally, some antibodies, both monoclonal and polyclonal, are capable of recognizing a broader spectrum of MC, rather than being limited to a specific variant. Several studies, such as those by Pírez et al. [1], Metcalf et al. [23] and Fischer et al. [24], have reported strong reactivity from polyclonal antibodies toward most MC variants. Monoclonal antibodies have also shown high reactivity with most MC variants, as reported in studies by Devlin et al. [7], Zeck et al. [25], Weller et al. [8] and Khreich et al. [21]. However, polyclonal antibodies raised against MC-LR by Metcalf et al. [23] were found to be less sensitive than those described by Young et al. [26]. Some of these antibodies also recognize demethylated variants at certain positions. In fact, demethylation of MC-RR at position 3 led to an increase in antibody sensitivity [8,26]. However, the results for MC-LR and MC-RR demethylated at this position, reported by Zeck et al. [25], Zeck et al. [15] and Metcalf et al. [23] were different, showing a decrease in sensitivity, although antibody recognition was still maintained. As previously mentioned, comparison between studies is difficult, as CRF values may depend on several experimental parameters simultaneously.

These antibodies, which exhibit high sensitivity to the most common MC variants,and not only those with an arginine at position 4, are developed against the conserved region known as the Adda moiety. This structural feature is critical for antibody recognition, not only of MC-LR but also of other variants [1,2,5,7,8,11,13,15,21,22,24-29]. Khreich et al. [21] studied this Adda recognition with the MC159 antibody, the monoclonal antibody strongly binds MC-LA, MC-YR, nodularin, and also Adda analogues. This study shows that this region is the most important for recognition by this antibody, leading us to believe that it may also be crucial for antibodies that recognize a broad spectrum of MCs, which only share this region in common.

Taken together, these findings support the conclusion that, while most antibodies exhibit the highest affinity for MC-LR, many are still capable of detecting other variants. This is an important consideration if ELISA is to be used as a method to detect more than one type of MC in a sample.

On the opposite side, some antibodies may not show this broad recognition capability. For example, the MC8C10 monoclonal antibody described by Sheng et al. [6], exhibits

high sensitivity toward MC-LR but it shows significantly lower reactivity to MC-RR and MC-YR, and no recognition to other MC variants such as MC-LF, MC-LW or nodularin.

An alternative strategy to the use of monoclonal and polyclonal antibodies involves the exploitation of antibody fragments, as described by Zhang et al. [3], these engineered antibodies can be produced at industrial scale, rapidly purified and fused with a marker molecule for immunological detection effectively, all at low cost. While this approach can increase the specific sensitivity toward a particular variant, specifically MC-LR, it may also reduce recognition of other important variants. Nevertheless, several studies using different antibody fragments have demonstrated good reactivity against the most common and potentially most toxic MC variants (i.e., MC-RR and MC-YR) [3,11,13]. Supporting this, Xu et al. [29] describe an antibody fragment that mirrors the reactivity pattern observed for the antibody MC10E7, recognizing preferentially variants with arginine at position 4, including MC-RR and MC-YR. A different cas is described by Murphy et al. [2], who reported an antibody fragment capable of recognizing a broad range of variants, including MC-YR, MC-LA, MC-LW, MC-LF, MC-RR, and even nodularin. However, its reactivity was lower than that of the monoclonal and polyclonal antibodies described in previous studies.

Due to the importance of the antibody production process in the recognition of toxins, a study compared two antisera (i.e., polyclonal antibodies): antiserum produced against MC-LR and antiserum produced against MC-RR. This study showed better cross-reactivity and lower limits of quantification for most variants and nodularin when using the MC-RR antiserum [26]. As MC-RR antiserum is more sensitive than that raised against MC-LR, these polyclonal antibodies are likely more effective for detecting low MC concentrations directly from environmental samples [1,5,11,12,23,24,26].

Regarding the assay format, no clear pattern has been observed that indicates their impact on the recognition of MC variants by different antibodies. It is important to recognize that this factor contributes to the variability in CRF values between studies, however it is not possible to affirm whether a method increases or decreases sensitivity, as other experimental parameters, such as the type of antibody used and other experimental conditions, are also important factors that may play a role in this recognition. To further elucidate this issue, experiments performed under the same conditions but only changing the format, should be undertaken. The same happens with the detection method, although we may hypothesise that its influence on the antibody recognition is lower (as the signal measurement is often performed after the competition step).

Overall, these factors that generate differences in antibody sensitivity and specificity have important implications for selecting the most appropriate antibody. The choice will depend on the analytical objective. For example, if the objective is related to human and animal health protection it will be necessary to detect all potentially toxic

MC variants; conversely, for research purposes it may be interesting to identify all variants and with high precision.

4.5. Comparison of toxicity equivalency factors

TEFs are presented in Annex II and compiled from the bibliography. These values are derived from in vivo and in vitro toxicological studies, typically referencing MC-LR as the most common and highly toxic MC variant, which is assigned a TEF of 1. The intrinsic toxicity, and thus the TEFs of the different MC variants and nodularin vary considerably due to several factors, including mechanisms of cellular uptake and the structural characteristics of the different compounds. MCs are generally unable to cross cell membranes by passive diffusion, because they are large and hydrophobic molecules. Instead, they require specific transporters, namely OATPs (organic anions transporting polypeptides). Some studies, therefore, have focused on these transporters. Fischer et al. [30] found no toxicity in HEK293 cells without OATPs, confirming the essential role of these transporters in MC uptake and cytotoxicity. Consistently, Monks et al. [31] found that MC-LR and related variants showed potent cytotoxic activity in OATP1B1/1B3-transfected HeLa cells, in comparison with control cells.

Fisher et al. [30] also showed that the difference in toxicity between MC variants depends on how efficiently they are transported into the cell. It is thought that MC variants with comparable hydrophobic properties exhibit similar permeabilities, as a result, they would exhibit similar cytotoxicity [32]. Moreover, several studies highlight the relationship between differences in hydrophobicity and toxicity, showing greater susceptibility to the more hydrophobic variants, MC-LW and MC-LF, compared to MC-LR and MC-RR [30,31]. Fischer et al. [30] observed a 7 to 60 fold increase in toxicity for MC-LW and MC-LF compared to MC-LR. Fischer et al. [30] also reported greater toxicity for the MC-LW and MC-LF in primary human hepatocytes. MC-RR was reported as the least toxic variant in Fischer's study [30], and other studies, such as that of Ufelmann et al. [33], did not observe any cytotoxicity at all for this variant. These studies indicate that MC-LR is not the most toxic variant, contrary to common belief. This underscores the importance of considering different experimental parameters and assay types when evaluating toxicity.

Hydrophobicity, permeability, and thus cellular uptake, can be affected and differ in demethylated variants, as demonstrated by some studies [9,32,33]. The absence of a methyl group at these positions has been associated with increased cytotoxic activity for MC-LR, MC-YR, and MC-RR [32]. Two studies, Ufelmann et al. [33] and Shimizu K. (2014), agree that the lack of a methylation at Dha7 or Asp3 enhances the cytotoxicity of MC-LR. Both studies also observed similar toxicity levels for methylated and demethylated forms of MC-RR [32,33]. However, their results differ regarding [Dha7]MC-YR: Shimizu et al. [32] reported that demethylation results in higher toxicity,

while Ufelmann et al. [33] found similar results of EC_{50} values between [Dha7]MC-YR and MC-YR, so no significant change.

Ikehara et al. [34] also evaluated MC-LR, MC-RR and MC-YR, but its findings differ from those reported by Shimizu et al. [32] and Ufelmann et al. [33], as the absence of methylation was associated to a reduced cytotoxic activity for all three variants.

The models and methodologies used to determine the toxicity of MC variants and nodularin have an influence on the results obtained. Although most of the results mentioned above follow a similar trend, there are slight differences due to these factors. For example, the use of two different cell types transfected with OATPs yields slightly different results, suggesting that the concentrations required to induce cytotoxicity in HeLa cells are significantly lower than those needed for HEK293 cells. The study by Ufelmann et al. [33] is also noteworthy, as it compares human and rat hepatocytes, demonstrating the importance of the used model on the toxin transport, since the transporters differ between the two species. This difference is reflected in the variation in toxicity, with human hepatocytes being less sensitive to all tested variants, although in this case, the trend followed by the different variants remains the same in both species. The importance of the method used to measure toxicity is highlighted in the study by Ufelmann et al. [33], where EC_{50} values obtained via the resazurin assay were consistently lower than those from the LDH leakage assay. Both assays were performed in the same study and, therefore, in this case, comparison between results is straightforward.

Beyond specific assay methodologies, it is important to consider how in vitro assay results correlate with in vivo toxicity. In this regard, the in vivo studies generally follow a similar trend to most studies in cells. However, the data obtained from this type of assay are limited and only evaluate the toxicity of MC-LR, MC-RR and MC-YR. The differences observed in vitro for these variants reflect the respective LD50 values from intraperitoneal studies in mice, where MC-LR is more toxic than MC-RR and MC-YR [35]. In vivo studies are lacking for other MC variants, especially MC-LF and MC-LW, that appear more toxic in vitro studies, in order to determine whether they also exhibit greater toxicity than MC-LR in vivo.

Finally, it is also important to highlight the observed interindividual variability. This was clearly demonstrated in Fischer et al. [30], where distinct responses were observed between two donors whose hepatocytes were used, despite being matched by sex and age group. This variability has important implications for environmental and regulatory toxicology [30].

4.6. Comparison of inhibition equivalency factors

IEFs, presented in Annex III, represent the relative inhibitory potency of MC variants and nodularins on protein phosphatases. They are classified according to MC variant, enzyme (PP2A or PP1), species origin and method. These values are expressed in relation to a reference standard, i.e. MC-LR, which is assigned an IEF of 1. High IEFs indicate high potency, whereas low IEFs indicate low inhibitory potency. In contrast, IC_{50} is inversely related to IEF, meaning that low IC_{50} values correspond to high IEFs, and vice versa. This section explores the primary mechanism of MC toxicity on enzymes and discusses the different factors that may play a role in IEF values. The primary mechanism underlying the toxicity of MCs and nodularins involves the potent and specific inhibition of eukaryotic protein phosphatases 1 (PP1) and 2A (PP2A), leading to hyperphosphorylation of cellular proteins and disruption of vital cellular functions, ultimately causing hepatotoxicity [33]. Unlike ELISAs, PPIAs are not based on structural recognition but instead provide a functional response that reflects the binding affinity of the toxins to these proteins and their inhibitory capacity [10]. It is known that MCs inhibit PP1 and more strongly PP2A [9]. This suggests that the type of enzyme plays a role in the inhibition effect. In general, wild-type PP2A (PP2AWild) showed the lowest IC_{50} values, while recombinant (rPP2A) showed the highest ones. The regulatory subunit of PP2AWild may facilitate MC binding and thus enhance inhibition compared to the recombinant catalytic subunits of rPP1 and rPP2A [20]. Besides, that inhibitory potency is higher in bovine PP2A and rabbit PP1 than in human enzymes [33].

Mountfort et al. [36] demonstrated different responses to diverse variants, with the highest inhibition observed for MC-LR and nodularin (lowest IC_{50} values), and the lowest for MC-RR (highest IC_{50}). Several authors agree that MC-LR is the most potent variant in terms of inhibitory activity. The interaction with phosphatases is influenced by structural features. Wang et al. [37] highlights the hydrophobic interaction between the Adda residue and PP1. When comparing variants, MC-LR exhibits stronger hydrogen bonding interactions. However, the total number of hydrogen bonds between the variants and PP1 (MC-LF> MC-LA> MC-LR> MC-LW> MC-LY) does not match the observed inhibition sequence (MC-LR> MC-LF> MC-LA> MC-LY> MC-LW). Most variants have an IEF <0.5, indicating lower inhibition compared to MC-LR. According to Garibo et al. [20], MC-LR is the most potent variant for all enzymes tested. In many cases, variants with arginine at position 4 (MC-LR, MC-RR and MC-YR) consistently show higher inhibition than other variants.

Several studies have reported alterations in inhibitory potency associated with structural modifications. In particular, demethylation at positions 3 and 7 results in reduced potency. This causes a greater decrease in phosphatase inhibition compared to amino acids substitutions at positions 2 and 4. These structural changes result in variants with lower inhibitory potency, especially against PP2A, and the effect is similar

whether both positions are demethylated together or individually [9,34, 36]. Further mechanistic insights into this demethylation effect are provided by Hoeger et al. [38], who demonstrated that MC variants lacking MDha at position 7 do not bind covalently to phosphatases, which results in a significant diminution of their inhibitory potency. This is evident when comparing the inhibition values of MC-RR and [Dha7]MC-RR. Similarly, Ufelmann et al. [33] notes that demethylation at [7Dha] reduces inhibitory activity in some variants, although this is not the case with MC-YR, indicating variant-specific effects. However, not all studies on the impact of demethylation on inhibitory potency are consistent. Some authors, such as Ufelmann et al. [33] and Hoeger et al. [38], report little to no effect of demethylation on inhibitory potency. These discrepancies underscore the complexity of structure-activity relationships and the influence of different assay conditions.

4.7. Comparison of the three factors

In the preceding sections, we have explored in detail the CRFs −which reflect the affinity of antibodies in immunoassays for different MCs and nodularin−, the TEFs −which measure the toxicity of different variants−, and the IEFs −which measure the specific inhibitory potency on protein phosphatases−. This final section critically examines the possible connections between these three factors and discusses the observed discrepancies.

As previously mentioned, the main mechanism of toxicity of MCs is attributed to their potent inhibition of protein phosphatases (PP1 and PP2A). Logically, one would expect a strong correlation between inhibitory potency (reflected in the IEFs) and toxicity (reflected in the TEFs). However, there is no agreement among the findings on whether there is a correlation between PP inhibition and MC cytotoxicity.

Some authors report a weak correlation between MC cytotoxicity and their ability to inhibit protein phosphatases (PP) [35,37,39]. For instance, Ikehara et al. [9] suggests that PP2A inhibition is more comparable to the mouse bioassay than the cytotoxic assay, in agreement with Chen et al. [40], who found a very good correlation between MCs toxicities in mice and the inhibitory activity against PP2A. Similarly, Monks et al. [31] data suggests that MC cytotoxicity in HeLa cells is related to specific PP2A inhibition, although found no correlation between global phosphate inhibition and cytotoxicity. As previously mentioned, the ADDA region present in MC and nodularin is important not only for the PP2A inhibition activity, but also for cytotoxicity. Moreover, Ufelmann et al. [33] revealed that non-cytotoxic variant MC-RR was 10- to 100- fold less potent inhibitor than the cytotoxic variant MC-LR and MC-YR, supporting the possible correlation between inhibition and cytotoxicity [9].

However, other studies have reported significant discrepancies. Ufelmann et al. [33] also revealed that the most potent human PP inhibitor was not the most cytotoxic variant, suggesting that factors other than PP inhibition may influence toxicity. Fischer

et al. [30] observed that, although many MCs show comparable inhibition of PPs in vitro, their LD_{50} values in mice differ significantly, indicating that in vitro findings do not necessarily reflect in vivo effects [38]. This suggests that toxicity differences may not be related to PP inhibition, but rather to how each variant is absorbed and distributed in the body.

Although the relation between toxicity and PP inhibition remains unclear, PPIAs are easier to perform and may serve as useful indicators of potential toxicity. In Garibo et al. [20], the PPIAs developed would not produce false negatives related to toxicity, only false positives, which would not pose a significant concern for public health.

When analysing samples, it is essential to link detection with toxicological relevance. If analysis is performed with instrumental techniques such as LC-MS/MS, the MC variants are identified and the TEFs or IEFs can be applied to the individual quantifications in order to estimate the total toxicity or inhibition potency of a sample. On the contrary, when immunochemical assays are used, the provided content will be based on a structural recognition. In an ideal situation and in order to protect animal and human health, the antibody should be able to detect the toxic MC variants. Although general trends indicate that there is a correlation, some discrepancies may arise. MC-RR is a clear example of this discrepancy, as it shows variable CRF values, depending on the method, and therefore, its detection can be either very good or very poor; and it has low TEF values, meaning that detection methods yielding high CRF values may lead to an overestimation of its toxicity. In terms of human health protection, this would not pose a problem. A different example is that of nodularin, which is generally a potent phosphatase inhibitor, but not all immunoassays exhibit high reactivity to it. Similarly, variants such as MC-LF and MC-LW tend to show low reactivity in most immunoassays, while possessing high TEF values, those posing a significant risk of underestimation. In these cases, immunoassays would not be appropriate.

5. CONCLUSIONS

In this work, the CRFs, IEFs and TEFs for MC variants reported in the literature as well as the CRFs experimentally obtained with a magnetic bead-based immunoassay, have been reviewed and compared, within each group and among them. The entire analysis clearly suggests that there is no generalized ideal correlation between CRFs, IEFs and TEFs for MC variants and nodularin.

There is a high intrinsic variability. The values for the same variant can vary within the same factor (CRF, IEF, TEF), depending on the antibody, the protein phosphatase, the species or the in vivo model used, the assay format or the detection technique, among other experimental parameters.

Additionally, despite some common trends, there are discrepancies with the phosphatase inhibition and the toxicity of MC variants. This indicates that while PP

inhibition is the principal mechanism of toxicity, other factors, like pharmacokinetics, may play a determinant role in vivo toxicity. A toxin may be an excellent inhibitor in vitro, but if it does not reach the target tissue, its toxicity will be very low.

There are also discrepancies when comparing the analytical detection capacity through immunoassays and toxicity. In this case, there is a risk of false negatives, reflecting an underestimation of risk in variants that are highly toxic (high TEF) but are not effectively detected (low CRF); and the risk of false positives, reflecting an overestimation of risk if a variant is easily detected (high CRF) but has low toxicity (low TEF).

In summary, the comparative analysis of CRFs, TEFs and IEFs for various MC variants and nodularin underscores the complexity of their detection, toxicity and inhibitory assessment. While immunochemical methods vary widely depending on the antibody type, the assay format and the detection method, toxicological potency is influenced by cellular uptake mechanisms, especially hydrophobicity and transporter specificity. Enzyme inhibition studies further highlight structural determinants, mainly the Adda moiety and demethylation, as key factors in the inhibition potency. Despite variability across studies, some consistent patterns emerge that can guide the selection of analytical tools, which will depend on the final purpose and application.

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ANNEX I: CRF values of MCs and nodularins compiled from this study and the bibliographic review

Ab: Antibody; **pAb**: polyclonal Antibody; **DC-ELISA**: Direct Competitive ELISA; **IC-ELISA**: Indirect Competitive ELISA; **SPR**: surface plasmon resonance; **FELISA**: Fluorescent ELISA ; **IC-CLEIA**: Indirect Competitive Chemiluminescent Enzyme Immunoassay

Variant	Antibody			Method	CRF	Ref.
MC-RR	Anti MC-LR	Monoclonal	Abraxis kit Ab	DC-ELISA	0,53	27
			5C4	DC-ELISA	1,08	7
			AD4G2	DC-ELISA	0,7	25
			M8H5	DC-ELISA	1,14	8
			Chu Ab	DC-ELISA	1,26	22
				IC-ELISA	0,704	28
			MC8C10	IC-ELISA	0,075	6
			M1B Ab	DC-ELISA	1,3	5
			M1C Ab	DC-ELISA	1	5
			M1D Ab	DC-ELISA	4,5	5
			M1E	DC-ELISA	5	5
			MC10E7	DC-ELISA	1,01	15
				DC-ELISA	0,779	This study
				SPR	0,88	4
		Polyclonal	AffiPoly Ab	DC-ELISA	1,1	5
			McElhiney Ab	IC-ELISA	0,68	1
			Young Ab	IC-ELISA	0,365	26
			AB824	IC-ELISA	0,5	24
			Xu pAb	IC-ELISA	1,36	11
			Luo pAb	FELISA	0,156	12
		Fragment	MscFv7	IC-ELISA	0,93	29
			scFv-2G1	SPR	0,601	2
			scFv-AP	IC-CLEIA	0,0215	3
			AVHH−MVH	IC-ELISA	1,225	13
			RscFv3	IC-ELISA	0,97	11
	Anti MC-RR	Polyclonal	Young antisera	IC-ELISA	1	26

MC-YR	Anti MC-LR	Monoclonal	Abraxis kit Ab	DC-ELISA	0,64	27
			5C4	DC-ELISA	0,68	7
			AD4G2	DC-ELISA	1,2	25
			M8H5	DC-ELISA	1,18	8
			Chu Ab	DC-ELISA	0,65	22
				IC-ELISA	0,513	28
			MC8C10	IC-ELISA	0,12	6
			MC159	DC-ELISA	0,92	21
				DC-ELISA	0,96	21
			M1B Ab	DC-ELISA	3,95	5
			M1C Ab	DC-ELISA	11	5
			M1D Ab	DC-ELISA	16	5
			M1E	DC-ELISA	4	5
			M2.1 Ab	DC-ELISA	5,1	5
			MC10E7	DC-ELISA	0,71	15
				DC-ELISA	0,516	This study
				SPR	0,94	4
		Polyclonal	AffiPoly Ab	DC-ELISA	2	5
			McElhiney Ab	IC-ELISA	0,68	1
			Young Ab	IC-ELISA	1,32	26
			AB824	IC-ELISA	1,67	24
			Xu pAb	IC-ELISA	0,92	11
		Fragment	MscFv7	IC-ELISA	0,86	29
			scFv-2G1	SPR	0,797	2
			scFv-AP	IC-CLEIA	0,0142	3
			AVHH−MVH	IC-ELISA	0,902	13
			RscFv3	IC-ELISA	0,56	11
	Anti MC-RR	Polyclonal	Young antisera	IC-ELISA	1,1133	26
MC-WR	Anti MC-LR	Monoclonal	AD4G2	DC-ELISA	0,76	25
			M8H5	DC-ELISA	1,47	8
			MC10E7	DC-ELISA	0,95	15
				DC-ELISA	0,69	This study
		Polyclonal	Xu pAb	IC-ELISA	1,19	11
		Fragment	MscFv7	IC-ELISA	<0,001	29
			scFv-AP	IC-CLEIA	<0,0001	3
			AVHH−MVH	IC-ELISA	0,029	13
			RscFv3	IC-ELISA	0,85	11

MC-LF	Anti MC-LR	Monoclonal	Abraxis kit Ab	DC-ELISA	0,72	27
			5C4	DC-ELISA	0,68	7
			AD4G2	DC-ELISA	0,69	25
			M8H5	DC-ELISA	1,23	8
			MC8C10	IC-ELISA	<0,000004	6
			MC10E7	DC-ELISA	<0,000004	15
		Polyclonal	McElhiney Ab	IC-ELISA	0,77	1
			Metcalf antisera	IC-ELISA	0,925	23
			Young Ab	IC-ELISA	0,6379	26
			AB824	IC-ELISA	1,08	24
			Xu pAb	IC-ELISA	<0,001	11
			Luo pAb	FELISA	<0,001	12
		Fragment	scFv-2G1	SPR	0,6749	2
			scFv-AP	IC-CLEIA	0,0001	3
			AVHH−MVH	IC-ELISA	0,001	13
	Anti MC-RR	Polyclonal	Young antisera	IC-ELISA	0,783	26
MC-LW	Anti MC-LR	Monoclonal	Abraxis kit Ab	DC-ELISA	1,02	27
			5C4	DC-ELISA	0,71	7
			AD4G2	DC-ELISA	0,84	25
			M8H5	DC-ELISA	1,04	8
			MC8C10	IC-ELISA	<0,000004	6
			MC10E7	DC-ELISA	<0,000004	15
		Polyclonal	McElhiney Ab	IC-ELISA	0,52	1
			Metcalf antisera	IC-ELISA	0,5	23
			Young Ab	IC-ELISA	0,6286	26
			AB824	IC-ELISA	1,18	24
			Xu Ab	IC-ELISA	0,065	11
			Luo Ab	FELISA	<0,001	12
		Fragment	scFv-2G1	SPR	0,6375	2
			scFv-AP	IC-CLEIA	0,0012	3
			AVHH−MVH	IC-ELISA	0,056	13
			MscFv7	IC-ELISA	0,097	29
			RscFv3	IC-ELISA	0,009	11
	Anti MC-RR	Polyclonal	Young antisera	IC-ELISA	0,4071	26

MC-LY	Anti MC-LR	Monoclonal	Abraxis kit Ab	DC-ELISA	0,736	27
			AD4G2	DC-ELISA	1,03	25
			M8H5	DC-ELISA	1,22	8
			Chu Ab	DC-ELISA	0,044	22
			MC10E7	DC-ELISA	0,0007	15
		Polyclonal	Metcalf antisera	IC-ELISA	1,25	23
			Young Ab	IC-ELISA	0,4779	26
			Xu Ab	IC-ELISA	0,007	11
		Fragment	scFv-AP	IC-CLEIA	0,0001	3
			AVHH−MVH	IC-ELISA	0,001	13
	Anti MC-RR	Polyclonal	Young antisera	IC-ELISA	0,6291	26
MC-LA	Anti MC-LR	Monoclonal	Abraxis kit Ab	DC-ELISA	0,48	27
			5C4	DC-ELISA	0,69	7
			AD4G2	DC-ELISA	0,66	25
			M8H5	DC-ELISA	1,15	8
			Chu Ab	DC-ELISA	0,0193	22
			MC159	DC-ELISA	1,04	21
				DC-ELISA	0,85	21
			MC10E7	DC-ELISA	<0,000004	15
		Polyclonal	Metcalf antisera	IC-ELISA	1,25	23
		Fragment	scFv-2G1	SPR	0,7475	2
			scFv-AP	IC-CLEIA	0,0001	3

Nodularin	Anti MC-LR	Monoclonal	Abraxis kit Ab	DC-ELISA	0,76	27
			5C4	DC-ELISA	0,94	7
			AD4G2	DC-ELISA	1,63	25
			M8H5	DC-ELISA	1,02	8
			Chu Ab	DC-ELISA	0,478	22
			MC8C10	IC-ELISA	0,001	6
			MC159	DC-ELISA	0,9	21
				DC-ELISA	0,91	21
			M1C Ab	DC-ELISA	2	5
			M1D Ab	DC-ELISA	970	5
			M2.1 Ab	DC-ELISA	9,7	5
			MC10E7	DC-ELISA	0,06	15
				DC-ELISA	0,441	This study
		Polyclonal	AffiPoly Ab	DC-ELISA	1,5	5
			Metcalf antisera	IC-ELISA	0,438	23
			Young Ab	IC-ELISA	0,1939	26
			AB824	IC-ELISA	1	24
			Luo Ab	FELISA	0,058	12
		Fragment	scFv-2G1	SPR	0,6925	2
	Anti MC-RR	Polyclonal	Young antisera	IC-ELISA	0,2722	26
[D-Asp3] MC-LR	Anti MC-LR	Monoclonal	AD4G2	DC-ELISA	0,97	25
			M8H5	DC-ELISA	1,14	8
			M1B Ab	DC-ELISA	1	5
			M1C Ab	DC-ELISA	0,9	5
			M1D Ab	DC-ELISA	1,9	5
			M1E	DC-ELISA	1	5
			M2.1 Ab	DC-ELISA	1,4	5
			MC10E7	DC-ELISA	0,55	15
		Polyclonal	AffiPoly Ab	DC-ELISA	1	5
			AB824	IC-ELISA	1,57	24

[D-Asp3] MC-RR	Anti MC-LR	Monoclonal	AD4G2	DC-ELISA	1,09	25
			M1B Ab	DC-ELISA	1,1	5
			M1C Ab	DC-ELISA	1	5
			M1D Ab	DC-ELISA	4	5
			M1E	DC-ELISA	3,9	5
			M2.1 Ab	DC-ELISA	2,8	5
			MC10E7	DC-ELISA	1,38	15
		Polyclonal	AffiPoly Ab	DC-ELISA	1	5
			Metcalf antisera	IC-ELISA	0,5	23
			Young Ab	IC-ELISA	0,8608	26
			AB824	IC-ELISA	0,8	24
	Anti MC-RR	Polyclonal	Young antisera	IC-ELISA	1,0595	26
[D-Asp3] [Dhb7] MC-RR	Anti MC-LR	Monoclonal	AD4G2	DC-ELISA	0,51	25
			M8H5	DC-ELISA	1,56	8
			MC10E7	DC-ELISA	0,7	15

ANNEX II: TEF values of MCs and nodularins compiled from the bibliographic review

MBA: Mouse Bioassay; **CBA**: Cell Bioassay; **i.p**.: intraperitoneal; **CTG assay**: CellTiter-Glo (ATP-based cell viability assay); **RRA**: Resazurin Reduction Assay (resazurin-based cell viability assay); **LDH**: Lactate Dehydrogenase Leakage Assay (measure cytotoxicity and cell damage); **MTS**: colorimetric cell viability assay with MTS; **MTT**: colorimetric cell viability assay with MTT; **SRB assay**: Sulforhodamine B Assay (measure cell density); **HEK-OATP1B1**: Human Embryonic Kidney cells transfected with Organic Anion Transporting Polypeptide 1B1 (OATP1B1); **HEK-OATP1B3**: Human Embryonic Kidney cells transfected with Organic Anion Transporting Polypeptide 1B3 (OATP1B3); **HeLa-OATP1B**: HeLa cell line transfected OATP1B1; **HeLa-OATP1B3**: HeLa cell line transfected with OATP1B1

Variant	Assay		Cell type	Species	TEF	Ref.
MC-RR	MBA	i.p	-	mouse	0,183	35
	CBA	CTG assay	Hepatocytes	rat	<0,8	32
		RRA	Hepatocytes	rat	0,536	33
		LDH	Hepatocytes	rat	0,903	33
		MTS	Hepatocytes	human	0,0468	9
		MTT	Hepatocytes	human	0,0203	30
			Hepatocytes	human	0,0273	30
			HEK-OATP1B1	human	<0,00492	30
			HEK-OATP1B3	human	0,2029	30
		SRB assay	HeLa-OATP1B1	human	0,0013	31
			HeLa-OATP1B3	human	0,0672	31
MC-YR	MBA	i.p	-	mouse	0,389	35
	CBA	CTG assay	hepatocytes	rat	0,5405	32
		RRA	hepatocytes	rat	5,3069	33
		LDH	hepatocytes	rat	9,309	33
		MTS	hepatocytes	human	0,330	9
		SRB assay	HeLa-OATP1B1	human	0,0555	31
			HeLa-OATP1B3	human	0,8666	31
MC-WR	CBA	MTS	hepatocytes	human	0,1233	9
MC-LF	CBA	MTT	hepatocytes	human	6,8	30
			hepatocytes	human	41	30
			HEK-OATP1B1	human	19,279	30
			HEK-OATP1B3	human	69,486	30
		SRB assay	HeLa-OATP1B1	human	12,5	31
			HeLa-OATP1B3	human	43,333	31

MC-LW	CBA	MTT	hepatocytes	human	6,8	30
			hepatocytes	human	61,5	30
			HEK-OATP1B1	human	20,5769	30
			HEK-OATP1B3	human	64,275	30
		SRB assay	HeLa-OATP1B1	human	16,667	31
			HeLa-OATP1B3	human	78	31
MC-LA	CBA	MTS	hepatocytes	human	1,947	9
Nodularin	CBA	RRA	hepatocytes	human	1,212	33
			hepatocytes	rat	1,7179	33
		LDH	hepatocytes	rat	1,3378	33
[D-Asp3] MC-LR	CBA	CTG assay	hepatocytes	rat	3,687	32
		LDH	hepatocytes	rat	13,279	33
		RRA	hepatocytes	rat	19,852	33
			hepatocytes	human	5,920	33
[D-Asp3] MC-RR	CBA	CTG assay	hepatocytes	rat	<0,8	32
		RRA	hepatocytes	rat	<0,536	33
			hepatocytes	human	<0,903	33
[Dha7] MC-LR	CBA	CTG assay	hepatocytes	rat	3,687	32
		LDH	hepatocytes	rat	2,487	33
		RRA	hepatocytes	rat	2,836	33
			hepatocytes	human	0,744	33
		MTS	hepatocytes	human	0,987	9
[Dha7] MC-RR	CBA	CTG assay	hepatocytes	rat	0,150	32
		RRA	hepatocytes	rat	0,536	33
		LDH	hepatocytes	rat	0,903	33
		MTS	hepatocytes	human	0,0137	9
[Dha7] MC-YR	CBA	CTG assay	hepatocytes	rat	1,914	32
		MTS	hepatocytes	human	0,661	9
[D-Asp3] [Dhb7] MC-LR	CBA	CTG assay	hepatocytes	rat	6,0150	32
[D-Asp3] [Dhb7] MC-RR	CBA	CTG assay	hepatocytes	rat	0,1616	32
		MTS	hepatocytes	human	<0,0074	9
[1Asp]Nodularin	CBA	LDH	hepatocytes	rat	0,692	33
		RRA	hepatocytes	rat	1,072	33
			hepatocytes	human	0,1983	33

ANNEX III: IEF values of MC compiled from the bibliographic review

PP2A: Protein Phosphatase 2A; **rPP2A**: recombinant PP2A; **rPP2Ac**: recombinant PP2A catalytic subunit; **PP2Ac**: PP2A catalytic subunit; **PP1**: Protein Phosphatase 1; **rPP1**: recombinant PP1; **p-NPP**: p-nitrophenyl phosphate assay; **MUP**: 4-methylumbelliferyl phosphate assay; **cPPIA**: colorimetric Protein Phosphatase Inhibition Assay; **[33P] MBP**: Radioactively labeled Myelin Basic Protein; **PPIA:** Protein Phosphatase Inhibition Assay

Variant	Assay		Species	IEF	Ref.
MC-RR	PP2A	p-NPP	human	0,571	9
		p-NPP	human	0,18	20
		MUP	human	0,0051	33
		MUP	human	0,0126	36
		MUP	bovine	0,0204	33
		cPPIA	human	1	30
		cPPIA	human	0,75	38
		[33P] MBP	human	0,00818	31
	rPP2A	p-NPP	human	0,34	20
	rPP2Ac	p-NPP	human	0,072	34
	PP1	MUP	human	0,0125	33
		MUP	rabbit	0,1428	33
		cPPIA	human	0,8	30
		cPPIA	rabbit	1,9	38
		[33P] MBP	human	0,2029	31
	rPP1	p-NPP	human	0,14	20
MC- YR	PP2A	p-NPP	human	0,256	9
		p-NPP	human	0,04	20
		MUP	human	0,1	33
		MUP	human	0,2444	36
		MUP	bovine	2,3333	33
		[33P] MBP	human	0,0545	31
	rPP2A	p-NPP	human	0,06	20
	rPP2Ac	p-NPP	human	0,147	34
	PP1	MUP	human	0,3077	33
		MUP	rabbit	11	33
		[33P] MBP	human	0,0778	31
	rPP1	p-NPP	human	0,03	20
MC-WR	PP2A	p-NPP	human	0,179	9

MC-LF	PP2A	p-NPP	human	0,333	9
		p-NPP	human	0,01	20
		cPPIA	human	0,818	30
		[33P] MBP	human	0,6	31
	rPP2A	p-NPP	human	0,03	20
	rPP2Ac	p-NPP	human	0,5	34
	PP1	p-NPP	human	0,5909	37
	PP1	cPPIA	human	0,6667	30
	PP1	[33P] MBP	human	0,636	31
	rPP1	p-NPP	human	0,01	20
MC-LW	PP2A	p-NPP	human	0,281	9
	PP2A	p-NPP	human	0,01	20
	PP2A	cPPIA	human	0,818	30
	PP2A	[33P] MBP	human	0,75	31
	rPP2A	p-NPP	human	0,03	20
	rPP2Ac	p-NPP	human	0,421	34
	PP1	p-NPP	human	0,191	37
	PP1	cPPIA	human	0,6316	30
	PP1	[33P] MBP	human	0,4516	31
	rPP1	p-NPP	human	0,01	20
MC-LY	PP2A	p-NPP	human	0,02	20
	rPP2A	p-NPP	human	0,04	20
	PP1	p-NPP	human	0,3291	37
	rPP1	p-NPP	human	0,01	20
MC-LA	PP2A	p-NPP	human	0,199	9
	PP2Ac	PPIA	rabbit	0,9375	10
	PP1	p-NPP	human	0,4727	37
Nodularin	PP2A	p-NPP	human	0,059	9
	PP2A	MUP	human	0,1765	33
	PP2A	MUP	human	1,2222	36
	PP2A	MUP	bovine	0,3443	33
	PP2Ac	PPIA	rabbit	1,5	10
	rPP2Ac	p-NPP	human	0,0888	34
	PP1	MUP	human	0,1081	33
	PP1	MUP	rabbit	1,1340	33
MC-LL	PP2Ac	PPIA	rabbit	1	10
[D-Asp3] MC-LR	PP2A	MUP	human	0,4286	33
	PP1	MUP	human	0,3636	33

[D-Asp3] MC-RR	PP2A	p-NPP	human	0,107	9
	PP2A	MUP	human	0,0026	33
	PP2A	cPPIA	human	0,45	38
	PP1	MUP	human	0,0074	33
	PP1	cPPIA	rabbit	3,8	38
[Dha7] MC-LR	PP2A	p-NPP	human	0,192	9
	PP2A	MUP	human	0,3333	33
	PP2A	MUP	bovine	1,909	33
	PP1	MUP	human	0,16	33
	PP1	MUP	rabbit	2,037	33
[Dha7] MC-RR	PP2A	p-NPP	human	0,109	9
	PP2A	MUP	human	0,0045	33
	PP2A	MUP	bovine	0,0567	33
	PP1	MUP	human	0,05	33
	PP1	MUP	rabbit	0,132	33
dmMC-YR	PP2A	MUP	human	0,0125	33
	PP2A	MUP	bovine	1,4	33
	PP1	MUP	human	0,0138	33
	PP1	MUP	rabbit	5,7895	33
[D-Asp3] [Dhb7] MC-LR	PP2A	p-NPP	human	0,126	9
[D-Asp3] [Dhb7] MC-RR	PP2A	p-NPP	human	0,145	9
	PP2A	cPPIA	human	29,8	38
	PP1	cPPIA	rabbit	56,4	38