Human Glutathione Transferases Catalyzing the Conjugation of the Hepatoxin Microcystin-LR

ARTICLE *in* CHEMICAL RESEARCH IN TOXICOLOGY · JUNE 2011

Impact Factor: 3.53 · DOI: 10.1021/tx2000976 · Source: PubMed

4 AUTHORS:

Franca Maria Buratti
Istituto Superiore di Sanità
18 PUBLICATIONS **390** CITATIONS
SEE PROFILE

Simona Scardala
Istituto Superiore di Sanità
18 PUBLICATIONS **146** CITATIONS
SEE PROFILE

Enzo Funari
Istituto Superiore di Sanità
83 PUBLICATIONS **1,212** CITATIONS
SEE PROFILE

Emanuela Testai
Istituto Superiore di Sanità
102 PUBLICATIONS **2,127** CITATIONS
SEE PROFILE

Chemical Research in Toxicology

ARTICLE

pubs.acs.org/crt

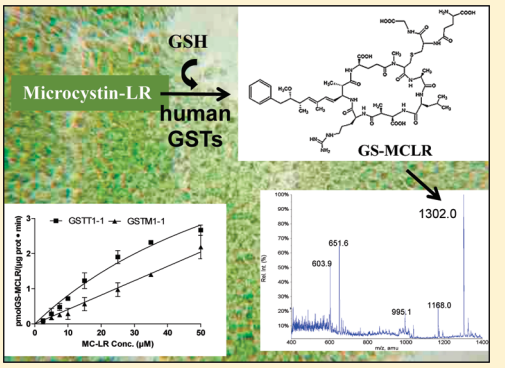
Human Glutathione Transferases Catalyzing the Conjugation of the Hepatoxin Microcystin-LR

Franca M. Buratti, Simona Scardala, Enzo Funari, and Emanuela Testai*

Environment and Primary Prevention Department, Istituto Superiore di Sanità, Viale Regina Elena 299, I-00161 Rome, Italy

Supporting Information

ABSTRACT: Many cyanobacterial species are able to produce cyanotoxins as secondary metabolites. Among them, microcystins (MC) are a group of around 80 congeners of toxic cyclic heptapeptides. MC-LR is the most studied MC congener, in view of its high acute hepatotoxicity and tumor promoting activity. Humans may be exposed to cyanotoxins through several routes, the oral one being the most important. The accepted pathway for MC-LR detoxication and excretion in the urine is GSH conjugation. The GSH adduct (GS-MCLR) formation has been shown to occur spontaneously and enzymatically, catalyzed by glutathione transferases (GSTs). The enzymatic reaction has been reported but not characterized both in vitro and in vivo in animal and plant species. No data are available on humans. In the present work, the MC-LR conjugation with GSH catalyzed by five recombinant human GSTs (A1-1, A3-3, M1-1, P1-1, and T1-1) has been characterized for the first time. All GSTs are able to catalyze the reaction; kinetic parameters K_m, k_{cat}, and their relative specific activities to form GS-MCLR were derived (T1-1 > A1-1 > M1-1 > A3-3 ≫ P1-1). In the range of MC tested concentrations used (0.25−50 μM) GSTT1-1 and A1-1 showed a typical saturation curve with similar affinity for MC-LR (≈80 μM; k_{cat} values 0.18 and 0.10 min^{-1}, respectively), A3-3 and M1-1 were linear, whereas GSTP1-1 showed a temperature-dependent sigmoidal allosteric curve with a k_{cat} = 0.11 min^{-1}. The enzymes mainly expressed in the liver and gastrointestinal tract, GSTA1-1, T1-1, and M1-1, seemed to be mainly involved in the MC-LR detoxification after oral exposure, whereas P1-1 kinetics and location in the skin suggest a role related to dermal exposure. Considering the high frequency of some GST polymorphism, especially M1 and T1 gene deletion, with complete loss in activity, this information could be the first step to identify groups of individual at higher risk associated with MC exposure.



1. INTRODUCTION

The nutrient overload from anthropogenic activities, determining eutrophic conditions in water bodies, has favored in the last years the growth of photosynthetic bacteria, known as cyanobacteria, up to such an elevated density to result in blooms and scum. Many cyanobacterial species are able to produce cyanotoxins as secondary metabolites.[1] Among them, microcystins (MC) are a group of around 80 congeners of toxic cyclic heptapeptides produced by different cyanobacteria, including *Microcystis*, *Anabaena*, and *Planktothrix* spp.[2] Their structure is characterized by a common specific amino acid Adda [(2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid], *N*-methyl-dehydroalanine (Mdha), D-alanine, β-linked D-erythro-8-methylaspartic acid, and γ-linked D-glutamic acid (Figure 1); they differ from each other for amino acids substitutions in position 2 and 4 and other changes such as methylation/desmethylation (Figure 1).

MC-LR is the most studied MC congener, characterized by the presence of leucine and arginine as L-amino acids in positions 2 and 4 (Figure 1).[3] On the basis of LD_{50} values, MC-LR is considered among the most acutely hepatotoxic MC variant.[1] Humans may be exposed to cyanotoxins through several routes: The oral one is by far the most important, occurring by ingestion of contaminated drinking water or food (including dietary supplements) or water during recreational activities. In addition, dermal/inhalation exposure may be associated with the domestic use of water (i.e., during a shower) or with professional and recreational activities (i.e., fishing). The parenteral route of exposure may also occur, when water from contaminated superficial water bodies is used for hemodialysis. This route of exposure is particularly dangerous and probably underestimated. In Brazil, 56 out of 130 hemodialyzed patients died, after treatment with MC-contaminated water.[4] Finally, a role for cyanobacteria in the etiology of amyotrophic lateral sclerosis (ALS) has been proposed.[5]

MC-LR mechanism of action is associated with specific inhibition of protein serine/threonine phosphatases (PP1 and PP2A), which alters phosphorylation of cellular proteins involved in signal transduction; this results in a cascade of events also described in human cell lines,[6] ranging from lipid peroxidation and oxidative stress to apoptosis.[7] Furthermore, in line with the known role of PP1 and PP2A in tumor suppression, MC-LR has been shown to have tumor-promoting activity;[8] on this basis, it

Received: March 3, 2011
Published: April 19, 2011

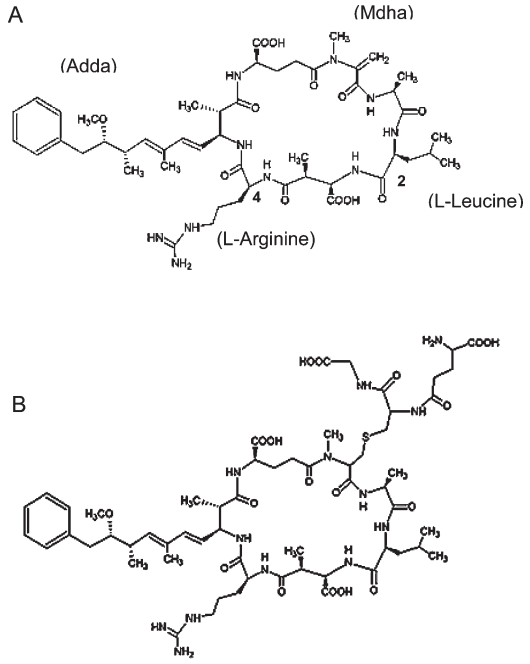


Figure 1. Chemical structure of MC-LR (A) and its GSH conjugate (GS-MCLR) (B).

was classified as a 2B carcinogen by the International Agency for Research on Cancer (IARC).[9]

The degree of severity of MC-LR-induced toxicity depends on the levels and duration of internal exposure, determined by the balance between absorption, detoxification, and excretion. Following MC-LR dosing, glutathione (GSH) depletion has been described, suggesting the possibility that the thiol is involved in MC-LR biokinetics. Indeed, the accepted pathway for MC-LR detoxication and excretion in the urine is GSH conjugation.[10−12] The GSH adduct (GS-MCLR) is obtained through nucleophilic reaction of the thiol to the α,β-unsaturated carbonyl of the Mdha moiety (Figure.1); its formation has been shown to occur spontaneously at alkaline pH [13] and enzymatically catalyzed by glutathione transferases (GSTs). On the basis of the LD_{50} values obtained after intravenous injection to mice, GSH conjugates showed a significant reduction in toxicity as compared with the parent compound.[13] The enzymatic reaction has been reported both in vitro with cell extracts from aquatic organisms and rats[14,15] and in vivo in invertebrates, plants, mussels, fish, and rodents.[10,11,16−18] The reaction has not been characterized in any of the species tested so far, and no data are available on humans.

Human GSTs are a large family of conjugating enzymes consisting of different classes (A, M, P, S, T, Z, O), some of them including several enzymes (1−5).[19] They catalyze nucleophilic attack by reduced GSH on a wide variety of nonpolar electrophilic compounds and thereby facilitate their elimination from the body. Within the cell, GSTs are mainly present as cytosolic enzymes, although a mitochondrial soluble form and a membrane-bound microsomal form also exist. They are widely distributed in different tissues, including the gastrointestinal (g.i.) tract, the liver, and the skin, which represent the main ports of entry following human exposure to MC. Several human GSTs show genetic functional polymorphisms, which alter the detoxification of a wide range of xenobiotics: Indeed, the polymorphism of GSTM1-1, P1-1, and T1-1 has been extensively studied as possible source of toxicokinetic variability and often associated to increased disease and cancer risk.[20]

The lack of information about the capability of human GST to catalyze MC conjugation limits the understanding of possible species differences and the relevance of the animal models used in toxicological tests. To fill the gap, the aim of the present work was to characterize the kinetic parameters of MC-LR conjugation with GSH by five recombinant human GSTs (A1-1, A3-3, M1-1, P1-1, and T1-1). Such information could contribute to the identification of metabolic biomarkers of individual susceptibility to MC toxicity, as the first step to identify groups of individual at higher risk related to MC exposure.

2. MATERIALS AND METHODS

2.1. Chemicals. MC-LR (purity >95%) isolated from *Microcystis aeruginosa* were obtained from Alexis-Enzo Life Sciences (Farmingdale, NY). Serva-Feinbiochemica (Heidelberg, Germany) supplied reduced GSH. All other analytical grade chemicals were obtained from commercially available sources.

2.2. Recombinant Human GSTs. Recombinant single GST (A1-1, A3-3, M1-1, P1-1, and T1-1) expressed in *Escherichia coli* were purchased from Oxford Biomedical Research (Rochester Hills, MI). The protein content of different preparations was in the range of 1−2.5 mg/mL (purity ≥95%). The specific GSTs' activity was determined by the supplier with the model substrate 1-chloro-2,4-dinitro-benzene, which is conjugated by almost all GSTs, except GST-T1, whose activity was measured by using 4-nitrobenzyl chloride. The catalytic efficiencies expressed as unit/mg protein were as follows: 10 (GSTP1-1), 29.4 (GSTT1-1), 42.1−51.6 (GSTA1-1), 61 (GSTA3-3), and 68.9 (GSTM1-1). Because the activities strictly depend on the substrate used, protein content rather than enzymatic unit was used in this work to quantify enzyme concentration in the incubation mixture.

2.3. In Vitro MC-LR Conjugation with GSH. *Chemical GS-MCLR Adduct Formation.* The synthesis of GS-MCLR was carried out according to the method of Kondo[13] with minor modification. Briefly, samples containing 8 mM GSH and MC-LR (0.25−15 μM) in 5% potassium carbonate aqueous solution were incubated at 30 °C under shaking (80 cycles/min) for 3 h. The reaction products were then analyzed with HPLC and confirmed with LC-MS/MS, as described below.

HPLC Analysis. The identification and quantification of MC-LR and GS-MCLR were obtained by DAD-HPLC method using a Perkin-Elmer Series 200 Liquid Chromatograph, equipped with a Perkin-Elmer diode array LC 235 detector (λ = 240 nm) and a Restek Pinnacle C-18 DB reversed-phase column (length = 25 cm, diameter = 4.6 mm). A linear gradient from 60:40 MeOH:water [both containing 0.05% trifluoroacetic acid (TFA)] to 70:30 in 10 min (flow rate, 1 mL/min) was used. The samples were injected after diluting MC-LR in a solution 1:1 (v/v) MeOH: Na-phosphate buffer (pH 7) to simulate the final incubation conditions. Indeed, before the analysis, all of the incubation samples were diluted 1:1 with MeOH to achieve a higher signal.[21] The retention times were as follows: 8.4 min for the conjugate and 10.5 min for MC-LR. The MC-LR quantification was carried out using commercial standard to build a calibration curve (12 concentrations tested within the range 0.25−100 μM; R^2 = 0.992, LOD = 0.06 μM, LOQ = 0.2 μM, and CV = 3.4%).

Because a commercial standard for the conjugate GS-MCLR is not available, a calibration curve was obtained with the reaction product obtained in extreme alkaline condition, as described above, after checking the completeness of the reaction; seven concentrations were used within the range 0.25−15 μM (R^2 = 0.992, LOD = 0.03 μM, LOQ = 0.1 μM, and CV = 3.8%). The samples were neutralized and diluted 1:1 with acidified MeOH (6% 37 N HCl) before the analysis to simulate the final incubation condition.

dx.doi.org/10.1021/tx2000976 |*Chem. Res. Toxicol.* 2011, 24, 926–933

LC-MS/MS Analysis. To analyze the chemically formed GS-MCLR adduct, samples were loaded on a ODS solid phase extraction cartridge to remove K_2CO_3. Prior to sample loading, the cartridge was conditioned with MeOH and water (4 mL each) and then rinsed with 20% MeOH (4 mL). The GS-MCLR conjugate was eluted with 4 mL of MeOH, dried under a gentle nitrogen stream, and redissolved in 0.5 mL of acetonitrile:water (30:70) containing 0.1% (v/v) formic acid. LC-MS/MS analysis was carried out on the following systems: two Micro-Pump and an autosampler PE series 200 (Perkin-Elmer Inc., United States), coupled to a MDS-SCIEX API 2000 triple-quadrupole mass spectrometer, equipped with a Turbospray source (MDS Sciex, Canada). For the multiple reaction monitoring (MRM) experiment, separation was achieved on a Grace Vydac Everest C-18 150 mm × 2.1 mm i.d. 300A 5 μm column (Grace Davison Discovery Sciences, United States), injecting 5 μL of sample. The mobile phase consisted of acetonitrile and water both added 0.1% (v/v) formic acid; total flow = 200 μL/min. The linear gradient program was as follows (with time *t* expressed in min): t_0 A = 30%, t_1 A = 30%, t_{15} A = 95%, t_{16} A = 95%, t_{17} A = 30%, and t_{26} A = 30%. For the identification of the GS-MCLR, the transitions *m/z* 1302 $[M + H]^+ \rightarrow$ 135; *m/z* 651.7 $[M + 2H]^{2+} \rightarrow$ 135 were monitored, whereas the quantification of the residual MC-LR was carried out as previously described.[22]

The conjugate formation in the enzymatic standard incubation mixture was confirmed by full scan analysis: The sample mixture was continuously infused into the ESI source at a flow rate of 10 μL/min through an integrated syringe pump. The MS experiment in the range *m/z* 400−1400 was carried out in positive ion mode, by using the following source settings: 5000 V ion spray voltage, 200 V declustering potential, 300 V focusing potential, and 10 V entrance potential.

Nonenzymatic Incubation. The standard incubation mixture (final volume 125 μL) contained the following: 100 mM Na-phosphate buffer (pH 7), 2 mM GSH, and 20 μM MC-LR added as methanolic solution. To avoid MC-LR adsorption on plastics, borosilicate test tubes were used.[21,23] The incubations were carried out at 37 °C under shaking (80 cycles/min) for 6 h and stopped in ice. The nonenzymatic GS-MCLR adduct formation was characterized at different GSH concentrations (range, 1−5 mM), pH values (range, 6.5−7.2), and temperature (28, 30, and 37 °C).

Enzymatic Incubation. The standard incubation mixture (final volume, 125 μL) in borosilicate test tubes contained the following: 100 mM Na-phosphate buffer (pH 7), 2 mM GSH, GST [2.5 μg protein/mL (A1-1 and T1-1) or 5 μg protein/mL (A3-3, M1-1, and P1-1)], and MC-LR added as methanolic solution (range of final concentrations, 2.5−70 μM). The enzymatic incubations were carried out at 37 °C under shaking (80 cycles/min) for 6 h; the reaction was stopped in ice and stored at −20 °C, when not immediately analyzed. To verify the GSH consumption/oxidation during the reaction, the GSH content was tested in the standard incubation mixture (1−5 mM GSH and 20 μM MC-LR at 6 and 18 h, 37 °C, and pH 7) according to Ellman[24] with some modifications. Briefly, 50 μL of samples was added to Ellman reagent (1 mM EDTA, 1 mM DNTB, and 0.1 mM K-phosphate buffer (pH 7.27); the absorbance was then measured at 412 nm.

To verify the linearity of the reactions vs protein concentration and time and to determine the optimal pH conditions, enzymatic incubations for each single GST isoform were carried out at different protein concentrations (1, 2.5, 5, 7.5, and 10 μg protein/mL), incubation times (2, 4, 6, and 8 h), and different pH values (6.5, 6.8, 7, and 7.2): 2 mM GSH and 20 μM MC-LR were used in these experimental sets. Blanks, differing only for the absence of GST in the incubation mixture, were carried out in parallel with corresponding samples, to detect the spontaneous GS-MCLR formation, which was then subtracted to the total conjugate production to derive the contribution of the enzymatic reaction only. The MC-LR and GS-MCLR quantification were carried out with the HPLC-DAD method, as described above.

2.4. Data Analysis. The kinetic parameters K_m and k_{cat} were obtained from nonlinear regression fit curve (Michaelis−Menten plot, allosteric sigmoidal, and Hill plot for GSTP1-1) with GraphPad 5 Prism software. The specific activity (expressed as V_{max}/K_m) was obtained as the slope of the linear regression of the linear tract of enzymatic activity curves.

3. RESULTS

3.1. In Vitro MC-LR and GSH Conjugation. *3.1.1. Preliminary Studies.* The set up DAD-HPLC method allows us to identify and quantify MC-LR and GS-MCLR formed during the incubation in the presence of human recombinant GSTs (Figure 2A). The chemical reaction between GSH and MC-LR in extreme alkaline pH condition (5% K_2CO_3 solution) was evidenced by the decrease of MC-LR peak and the presence of a new peak; the peak was then identified as the GS-MCLR adduct by the full scan experiment in LC-MS/MS (Figure 2B), showing the presence of ions *m/z* 1302.8 and *m/z* 651.6, consistent with the $[M + H]^+$ and the doubly protonated ion $[M + 2H]^{2+}$ for GS-MCLR, formed via addition of GSH to MC-LR.[25] Other ions *m/z* were present including *m/z* 1168, tentatively identified as $[M + H\text{-Adda}]^+$,[25] and *m/z* 603, which are considered not to be of relevance in the context of this work. Both the MC-LR and the conjugate were found to be stable at extreme alkaline conditions.

When the reaction was carried out with low cyanotoxin concentrations (0.25−15 μM), MC-LR appeared to be completely converted into the conjugated form: The MRM analysis in LC-MS/MS indicated residual MC-LR levels from negligible to 2% maximum (Figure 2C). The enlarged region (2×) in Figure 2B shows the presence of ion at *m/z* 995, which can be assigned to the residual MC-LR in the mixture. Consequently, the reaction was considered complete, and the product obtained was used as a standard, to build up a specific calibration curve for conjugate quantification.

3.1.1.1. Characterization of the Spontaneous Reaction. The nonenzymatic reaction was linearly dependent on GSH in the range of concentration 1−5 mM (see the Supporting Information) and on pH values (6.5, 7, and 7.2), increasing rapidly at pH > 7. The reaction also increased with incubation temperature (data not shown).

3.1.1.2. Characterization of the Enzymatic Reaction. All tested recombinant GSTs were able to catalyze MC-LR conjugation. The enzymatic activity was dependent on pH: It increased up to pH 7, then it slightly declined (see the Supporting Information). When the dependence on pH of the enzymatic and spontaneous reaction was compared in our experimental conditions, similar or slightly higher production of enzyme-mediated GS-MCLR was obtained at pH 7.

After 6 h of incubation with 1, 2, 3, and 5 mM GSH (initial concentrations) and the different GSTs, 80−90% residual GSH content was obtained, whereas at longer times, the decrease was substantial (i.e., up to 90% after 18 h for 1 mM GSH). When tested at pH 7, the enzymatic reaction was linear up to 8 h of incubation time and 5−10 μg protein/mL (data not shown).

On this basis, 2 mM GSH as a nonlimiting concentration, pH 7, 37 °C (close to the physiological situation), 6 h, and 2.5−5 μg protein/mL were used as optimal incubation conditions, to minimize the spontaneous reaction and maximize the enzymatic GS-MCLR formation. However, blanks were carried out for each set of experiments, to subtract the spontaneous contribution to the total GS-MCLR formation.

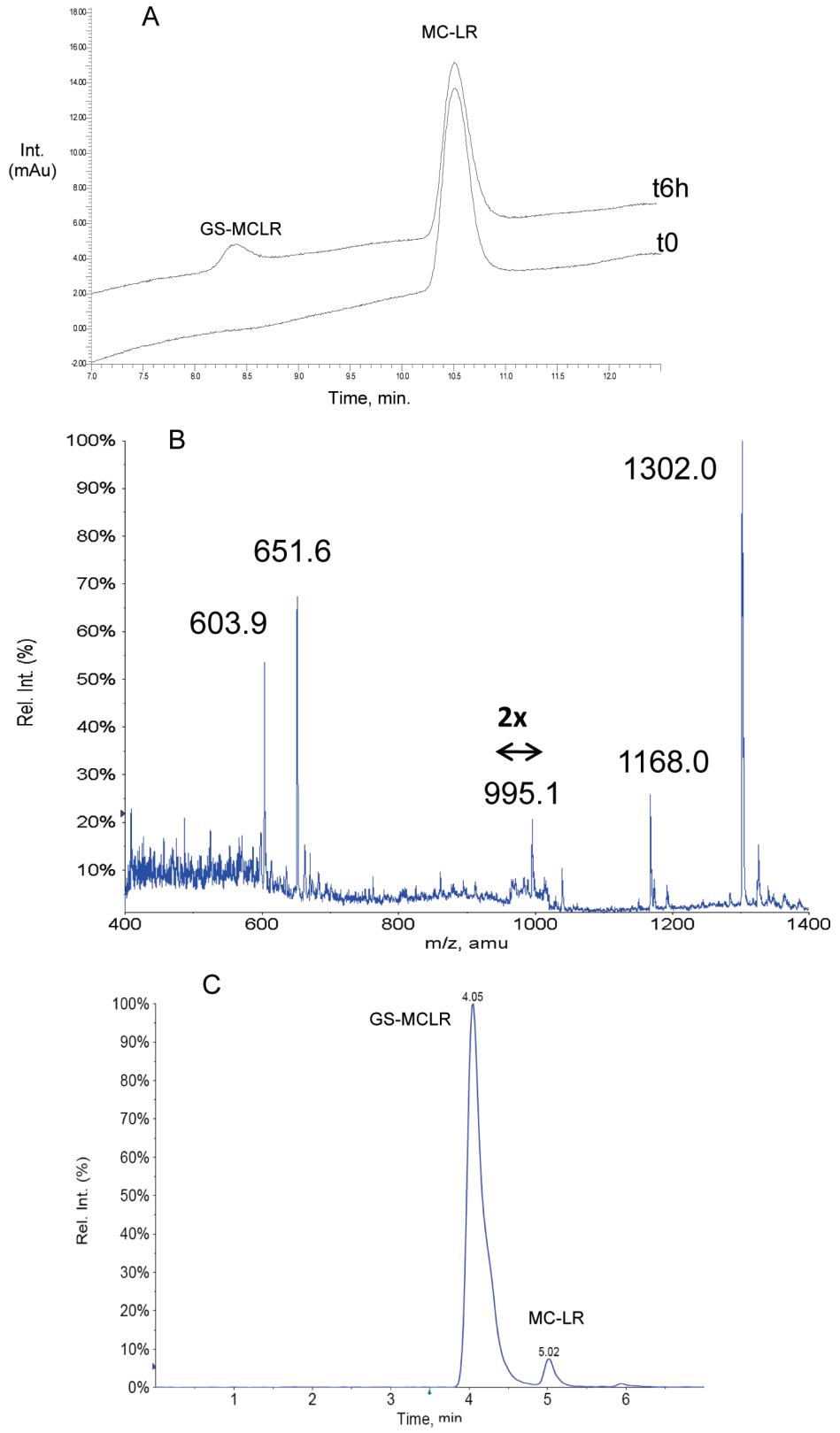


Figure 2. (A) HPLC chromatograms of MC-LR (retention time = 10.5 min) and GS-MCLR (retention time = 8.4 min) at the beginning of the enzymatic reaction (t_0) and at the end of the incubation (t_{6h}). Experimental conditions: 100 mM $NaPO_4$ buffer, pH 7, 2 mM GSH, 2.5 μg GST/mL, and 10 μM MC-LR, 37 °C. (B) Mass spectrum of the reaction products between MC-LR and GSH. Ions m/z 1302.8 $[M + H]^+$ and m/z 651.6 $[M + 2H]^{2+}$ correspond to GS-MCLR formed via addition of GSH to MC-LR. Enlarged region (2×): ion at m/z 995, assigned to the residual MC-LR in the mixture. (C) MRM analysis in LC-MS/MS: GS-MCLR and residual MC-LR peaks after chemical reaction (8 mM GSH and 0.25−15 μM MC-LR in 5% K_2CO_3 aqueous solution, 30 °C, 3 h).

dx.doi.org/10.1021/tx2000976 |*Chem. Res. Toxicol.* 2011, 24, 926–933

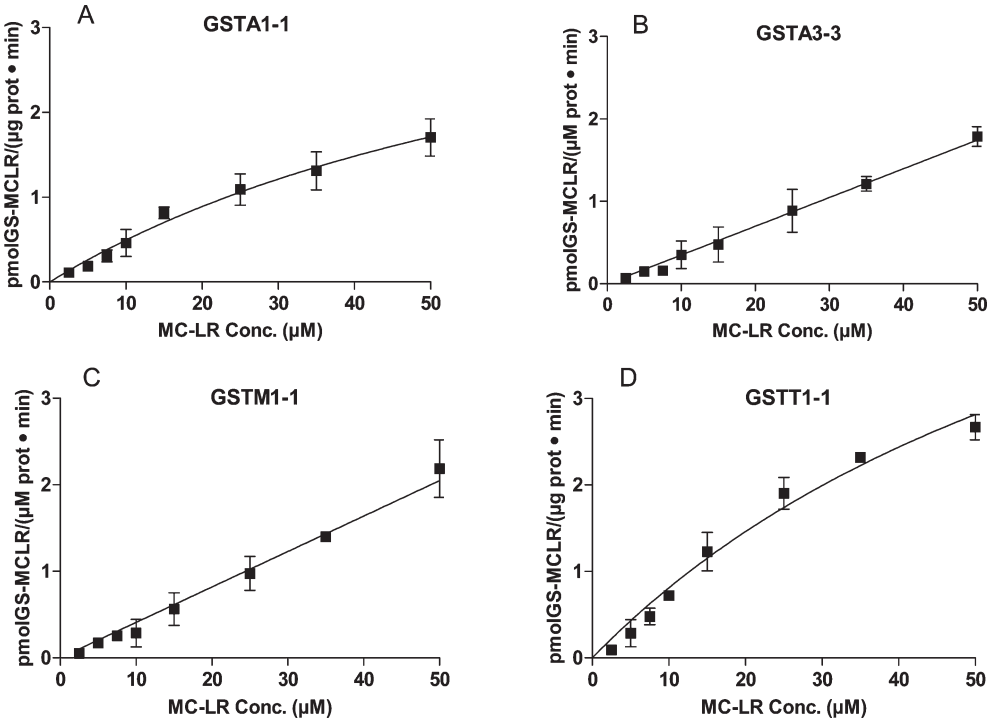


Figure 3. Rate of GS-MCLR enzymatic formation catalyzed by GSTA1-1 (A), GSTA3-3 (B), GSTM1-1 (C), and GSTT1-1 (D). Results are expressed as pmol GS-MCLR (μg prot min)$^{-1}$ and represent the mean ± SD on three independent determinations. Experimental conditions: 100 mM $NaPO_4$ buffer, pH 7, 2 mM GSH, 2.5−5 μg GST/mL, and 2.5−50 μM MC-LR, 37 °C, 6 h.

Table 1. Kinetic Parameters of GS-MCLR Formation by Human Recombinant GSTs[a]

	K_m[b]	V_{max}/K_m[c]	k_{cat}[d]	k_{cat}/K_m[e]
GSTT1-1	81 ± 23	0.075 ± 0.004	0.18 ± 0.05	0.0022
GSTA1-1	80 ± 29	0.049 ± 0.003	0.10 ± 0.02	0.0012
GSTM1-1	ND	0.041 ± 0.001	ND	ND
GSTA3-3	ND	0.035 ± 0.001	ND	ND

[a] Data represent means ± standard errors from at least three replicates. ND, not determined. [b] μM. [c] Values represent the slope of the linear trait of the Michaelis−Menten curve and are expressed as pmol GS-MCLR (μg prot min μM)$^{-1}$. [d] min^{-1}. [e] μM^{-1} min^{-1}.

3.1.2. Kinetic Parameters of GS-MCLR Formation. In the range of MC-LR concentration used (0.25−50 μM) GSTA1-1 and T1-1 showed a typical saturation curve, whereas A3-3 and M1-1 were linear at the maximum MC-LR concentration tested (Figure 3A−D). Table 1 reports the kinetic parameters K_m, k_{cat}, and V_{max}/K_m value indicating that the specific activity score is T1-1 > A1-1 > M1-1 > A3-3.

In the range of MC-LR concentrations tested (5−70 μM), GSTP1-1 showed a sigmoidal allosteric curve (Figure 4A). The atypical kinetic was shown to be temperature dependent: When the reaction was carried out at 30 °C, the sigmoidal allosteric shape of the curve was lost, and a typical Michaelis−Menten saturation curve was obtained (Figure 4A).

The experimental data obtained at 37 °C and the V_{max} value (4.2 ± 0.6 pmol GS-MCLR μg prot^{-1} min^{-1}) coming out from the allosteric model were used to draw a Hill plot (Figure 4B). From that plot, a Hill coefficient $n = 1.72 \pm 0.09$ giving the degree of sigmoidicity was derived as well as the K_{05} value of 585 ± 13 μM, corresponding to the substrate concentration resulting in 50% of V_{max} value (analogous to K_m in Michaelis−Menten equation).[26] The k_{cat} value is 0.11 min^{-1}. Similar results were obtained by raising to n the MC-LR concentration in the Hill equation; a Michaelis−Menten plot can be obtained (Figure 4C) ($K_m = 798 \pm 153$ μM).

4. DISCUSSION

In spite of the emergent health problem related to cyanotoxins exposure, the available toxicological information is limited, especially on humans, and risk assessment is possible only in few cases, with huge degrees of uncertainties.[1] The U.S. EPA has indicated the role of metabolism and particularly the characteristics of human toxicokinetics, among the major research needs.[27] Nevertheless, this kind of information is still very scant. In the present paper, we report for the first time the capability of human recombinant GSTs to catalyze the GSH conjugation of MC-LR, considered as the major detoxication pathway of MCs. In addition, although the formation of the GSH conjugate of MC-LR has been found in different organisms,[10,13,14] the catalytic efficiency of the conjugation reaction was never determined so far. Here, we characterized the kinetics of selected human c-DNA expressed GSTs deriving biochemical parameters indicative of enzyme affinities, specific activity or catalytic efficiencies. This approach leads to a better estimate of the relevance of each isoform in GSH conjugation, following exposure to MCs.

Because of lack of standard, up to now, the quantification of the GS-MCLR adduct has been carried out using the peak areas;[10,14] this approach was based on the quite similar absorption maximum, shown by the parent molecule and the conjugate (238 and 240 nm, respectively), which is due to the presence of the Adda moiety. In the present paper, a specific calibration curve

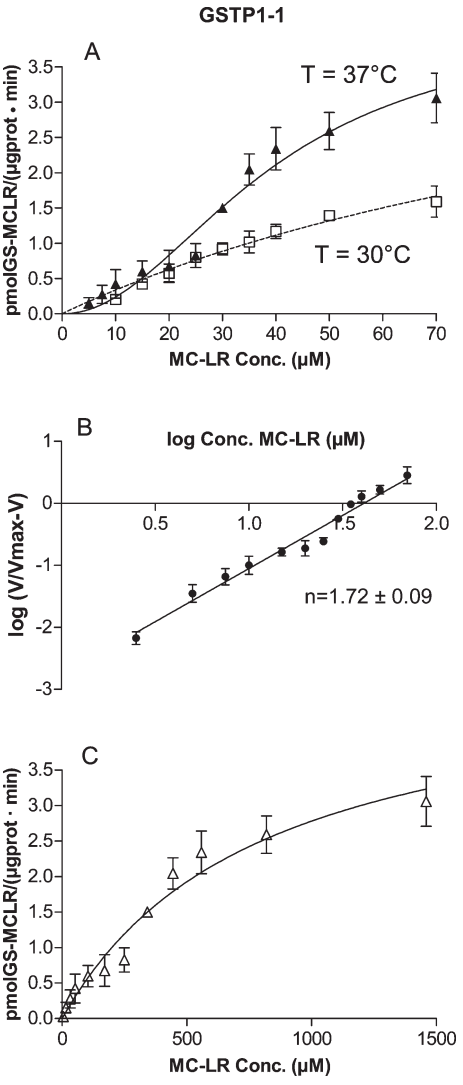


Figure 4. (A) Rate GS-MCLR enzymatic formation catalyzed by GSTP1-1 at 37 and 30 °C. Experimental condition: 2 mM GSH, 5−70 μM MC-LR, 37 or 30 °C, 6 h of incubation time, and 5 μg GST/mL. (B) Hill plot $\{\log[v - (V_{max} - v)]$ vs $\log S\}$, in which the value of the Hill coefficient n (degree of sigmoidicity) is also reported. (C) Michalis−Menten curve obtained by raising the MC-LR concentration to the Hill coefficient. Results are expressed as pmol GS-MCLR $(\mu g \text{ prot min})^{-1}$ and represent the mean ± SD of three independent determinations.

was obtained by using, as a standard, the product of the complete reaction between GSH and MC-LR in extreme alkaline conditions, identified by LC/MS-MS as the MC-LR conjugate. The ratio between the MC-LR and the GS-MCLR calibration curves was 1.4, evidencing that the use of peak areas leads to underestimate the conjugate formation.

In setting up the optimal experimental conditions, beside the physiological temperature typical for human enzymes (37 °C), we have used a GSH concentration (2 mM) lower than the one used in previous papers (around 8 mM). Indeed, it has been evidenced that enzymatic reaction rate can increase up to 1 mM GSH, remaining then constant at higher thiol concentrations.[28,29] In addition, the linear dependence of the spontaneous reaction on temperature and GSH content limits the use of high GSH concentrations. Previous data have been obtained at 28 °C, the physiological temperature for fish enzymes,[10,11,14] or at room temperature,[15] experimental conditions giving rise to a lower spontaneous formation of GS-MCLR adduct. Consequently, the choice of 2 mM GSH allowed minimization of the spontaneous GS-MCLR formation, it could guarantee saturation conditions for the enzymatic reaction,[29] remaining close to intracellular physiological GSH content (1−3 mM). The dependence on pH shown by GSTs' activity with several substrates,[28,30] reaching a maximum at pH 7, was confirmed with MC-LR.

Being that the oral route is highly relevant for human exposure to MC, the presystemic first pass metabolism occurs mainly in g.i. tract mucosa and in the liver; therefore, GSTs present in these tissues have been considered of particular interest and selected for testing. The distribution of the major GSTs subfamilies (GSTA, M, T, and P) in human hepatic and intestinal cytosol shows a high degree of variability.[31−35] However, GSTA1-1 and M1-1 are generally highly expressed in the liver: at similar levels according to some authors, whereas others indicated GSTA representing up to 84% of all hepatic GSTs (mainly as GSTA1).[31−34] In the g.i. tract mucosa, GSTP1 and A1 are present at high levels: GSTP1 shows a decrease of expression from stomach to colon, while GSTA is mainly expressed (up to 800-fold) in the small intestine.[35] GSTT1 is expressed in the liver, kidney, and erythrocytes, with lower levels in small intestine. Because during aquatic recreational activities the exposure to MC-LR is mainly dermal, GSTP1-1 was also selected for being the most abundantly expressed GST in the skin.[36]

All human GSTs tested were able to form the conjugate GS-MCLR. GSTT1-1 and GSTA1-1 showed a similar affinity for the cyanotoxin (expressed as K_m), with GSTT1-1 having the highest catalytic efficiency. Because GSTM1-1 was not saturated in the range of tested concentrations, it could have a higher relevance in case of acute exposure to high MC-LR levels, whereas at low MC-LR concentrations, likely representative of chronic exposure, GSTT1-1 and A1-1 could be mainly involved.

A temperature-dependent positive homotropic cooperativity was shown by GSTP1-1, determining a sort of autoactivation of the reaction at 37 °C, not present at 30 °C (Figure 4A). This atypical kinetic has been previously described as positive and negative cooperativity between GSH and other cosubstrates above 35 °C and below 25 °C, respectively.[37] It has been postulated that whenever a temperature variation induces a low-affinity conformation for GSH binding, GSTP1-1 opposes this perturbation by developing positive cooperativity, thus increasing the GSH affinity for the substrate monoligated enzyme.[37] This mechanism minimizes changes of GSH affinity for GSTP1-1 because of temperature fluctuation. The physiological advantage of the homotropic modulation mechanism is evident considering that GSTP1-1 is present in the upper layers of human epidermis, which may suffer low and high temperatures. Therefore, MC-LR detoxication seems to be favored at the exposure site in case of recreational activity in contaminated waters during summer, when the skin temperature reaches values higher than 35 °C. The affinity for the cyanotoxin was much lower when compared to the other GSTs, but the k_{cat} value (0.11 min^{-1}) was very similar.

Starting from catalytic efficiency values and individual GST tissue content, it would be possible to extrapolate the relative contribution of each GST, by following a consolidated approach, previously used for cytochrome P450 enzymes.[38,39] However, the extremely high variability limits the definition of GSTs' average tissue content, not allowing any quantitative estimate. In addition, our data, obtained with isolated enzymes, need to be confirmed on human cytosols or cells, where the different GSTs

are simultaneously present and collaborate with each other. Indeed, we have characterized only a few of them, selected on the basis of their localization, and others could be active. The use of more complex biological systems would allow understanding of the relative contribution and the relevance in vivo of the GST-catalyzed conjugation to the total MC-LR detoxication, to which the spontaneous reaction adds its role.

The evident high interindividual variability of GSTs content could also be attributed to the high frequency of GST polymorphism, especially when involving gene deletion with complete loss in activity.[20,40] About 40−60 and 15−25% of Caucasian population lacks M1 and T1, respectively,[20,39] with a frequency of 5−12% of "double null" M1 and T1 genotype.[20] Frequencies differ with ethnicity: As an example, in Asians, GSTT1 shows gene deletion in up to 60% individuals,[20,40] with a "double null" genotype frequency of 40%.[20] GSTP1 and GSTA also show functional polymorphism, with substrate-dependent activity alterations.[41]

Individuals characterized by "double null" mutations, endowed with a decreased level of GST activity, can be expected to experience a lower formation of conjugates and a longer half-life of the parent compound, resulting in a higher risk of MC-induced toxic effects. It is noteworthy to consider that (i) the polymorphisms across GST genes is absent in rodent and (ii) human cytosol has shown 3−4-fold lower activity than mouse cytosol for many substrates.[20] To have information related to MC-LR detoxication would allow one to know whether, and to which extent, results of animal toxicological tests, used in the risk assessment, are representative for the human population.

This is also important in view of the hypothesis about the role for cyanobacteria in the onset of ALS in susceptible individuals. Indeed, MC-LR is able to induce oxidative stress in vitro and in vivo, to alter the activity and/or the expression of related enzymes, including superoxide dismutase (SOD).[42−45] Because oxidative stress and SOD alterations are considered as possible risk factors associated to ALS onset, an altered detoxication, increasing the half-life of MC-LR within the organisms, is expected to interfere also through this kind of mechanisms.

Very few data are available to evaluate the eventual internal dose after MC-LR exposure in humans; free MC concentrations in serum have been measured in Brazil after two outbreaks in hemodialyzed patients: 0.2−0.96 μg/L (0.2−1 nM) was found in asymptomatic individuals,[46] whereas after the Caruaru episode, up to 28.8 μg/L (29 nM) was detected in serum from surviving patients.[47] Both measurements were done, respectively, 30−57 days and more than 2 months after the documented exposure. Total (free plus protein-bound) MC concentrations up to 112.9 μg/L (0.1 μM) were yielded in samples from six Caruaru patients (for which dates of collection were not available).[47] Therefore, considering that (i) immediately after the exposure the MC serum concentration was certainly higher, (ii) MC-LR is not expected to bioaccumulate, so that the amount detected likely represented the lower end elimination curve, and (iii) the concentration in the liver is generally higher than the circulating one; the MC-LR concentrations that we have used may be considered in the range of acute exposure. It can be anticipated that lower MC concentrations should be easily detoxified, although the presence of more susceptible individuals with different kinetics cannot be excluded.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Figures of dependence of the spontaneous GS-MCLR formation on GSH concentration and pH and dependence of the enzymatic GS-MCLR formation on pH. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +39 06 4990 2920. Fax: +39 06 49387139. E-mail: emanuela.testai@iss.it.

Funding Sources

This work has been partially supported by Grant R1-L from AISLA.

■ REFERENCES

(1) Funari, E., and Testai, E. (2008) Human health risk assessment related to cyanotoxins exposure. *Crit. Rev. Toxicol. 38*, 97–125.

(2) Chorus, L., and Bartram, J. (1999) *Toxic Cyanobacteria in Water*, Published on behalf of WHO by E&FN Spon, London, NY.

(3) Carmichael, W. W. (1998) Toxins of freshwater algae. *Handbook of Natural Toxins*, 121−147, M. Dekker, New York.

(4) Azevedo, S. M., Jochimsen, E. M., Rinehart, K. L., Lau, S., Shaw, G. R., and Eaglesham, G. K. (2002) Human intoxication by microcystins during renal dialysis treatment in Caruaru-Brazil. *Toxicology 181*, 441–446.

(5) Stipa, G., Taiuti, R., de Scisciolo, G., Arnetoli, G., Tredici, M. R., Biondi, N., Barsanti, L., and Lolli, F. (2006) Sporadic amyotrophic lateral sclerosis as an infectious disease: A possible role of cyanobacteria? *Med. Hypotheses 67*, 1363–1371.

(6) Botha, N., Gehringer, M. M., Downing, T. G., van de Venter, M., and Shephard, E. G. (2004) The role of microcystin-LR in the induction of apoptosis and oxidative stress in CaCo2 cells. *Toxicon 43*, 85–92.

(7) Gehringer, M. M. (2004) Microcystin-LR and okadaic acid-induced cellular effects: A dualistic response. *FEBS Lett. 557*, 1–8.

(8) Nishiwaki-Matsushima, R., Ohta, T., and Nisshiwaki, S. (1992) Liver tumor promotion by the cyanobacterial cyclic peptide toxin microcystin-LR. *J. Cancer Res. Clin. Oncol. 118*, 420–424.

(9) IARC (2006) Cyanobacterial peptide toxins. Available at http://monographs.iarc.fr/ENG/ Meetings/94-cyanobacterial.pdf.

(10) Pflugmacher, S., Wiegand, C., Oberemm, A., Beattie, K. A., Krause, E., Codd, G. A., and Steinberg, C. E. W. (1998) Identification of an enzymatically formed glutathione conjugate of the cyanobacterial hepatotoxin microcystin-LR: the first step of detoxication. *Biochim. Biophys. Acta 1425*, 527–533.

(11) Pflugmacher, S., Wiegand, C., Beattie, K. A., Krause, E., Steinberg, C. E., and Codd, G. A. (2001) Uptake, effects, and metabolism of cyanobacterial toxins in the emergent reed plant *Phragmites australis* (cav.) trin. ex steud. *Environ. Toxicol. Chem. 20*, 846–852.

(12) Dittmann, E., and Wiegand, C. (2006) Cyanobacterial toxins—Occurrence, biosynthesis and impact on human affairs. *Mol. Nutr. Food Res. 50*, 7–17.

(13) Kondo, F., Ikai, J., Oka, H., Masano, O., Ishikawa, N., Harada, K.-I., Matsuura, K., Murata, H., and Suzuki, M. (1992) Formation, characterization and toxicity of the glutathione and cysteine conjugates of toxic heptapeptide microcystins. *Chem. Res. Toxicol. 5*, 591–596.

(14) Šetlíková, I., and Wiegand, C. (2009) Hepatic and branchial glutathione S-transferases of two fish species: substrate specificity and biotransformation of microcystin-LR. *Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol. 149*, 515–523.

(15) Takenaka, S. (2001) Covalent glutathione conjugation to cyanobacterial hepatotoxin microcystin LR by F344 rat cytosolic and microsomal glutathione S-transferases. *Environ. Toxicol. Pharmacol. 9*, 135–139.

(16) Pflugmacher, S., Wiegand, C., Werner, S., Schröder, H., and Kankaanpää, H. (2005) Activity and substrate specificity of cytosolic and microsomal glutathione S-transferase in Australian black tiger prawns (*Penaeus monodon*) after exposure to cyanobacterial toxins. *Environ. Toxicol. 20*, 301–307.

(17) Kondo, F., Matsumoto, H., Yamada, S., Ishikawa, N., Ito, E., Nagata, S., Ueno, Y., Suzuki, M., and Harada, K.-I. (1996) Detection and identification of metabolites of microcystins in mouse and rat liver. *Chem. Res. Toxicol. 9*, 1355–1359.

(18) Robinson, N. A., Pace, J. G., Matson, C. F., Miura, G. A., and Lawrence, W. B. (1991) Tissue distribution, excretion and hepatic biotransformation of microcystin-LR in mice. *J. Pharmacol. Exp. Ther. 256*, 176–182.

(19) Hayes, J. D., Flanagan, J. U., and Jowsey, I. R. (2005) Glutathione transferases. *Annu. Rev. Toxicol. Pharmacol. 45*, 51–88.

(20) Ginsberg, G., Smolenski, S., Hattis, D., Guyton, K. Z., Johns, D. O., and Sonawane, B. (2009) Genetic polymorphism in Glutathione Transferases (GST): population distribution of GSTM1, T1, and P1 conjugating activity. *J. Toxicol. Environ. Health, Part B 12*, 389–439.

(21) Hyenstrand, P., Metcalf, J. S., Beattie, K. A., and Codd, G. A. (2001) Effects of adsorption to plastics and solvent conditions in the analysis of the cyanobacterial toxin Microcystin-LR by high performance liquid chromatography A. *Water Res. 35*, 3508–3511.

(22) Manganelli, M., Scardala, S., Stefanelli, M., Vichi, S., Mattei, D., Bogialli, S., Ceccarelli, P., Corradetti, E., Petrucci, I., Gemma, S., Testai, E., and Funari, E. (2010) Health risk evaluation associated to *Planktothrix rubescens*: An integrated approach to design tailored monitoring programs for human exposure to cyanotoxins. *Water Res. 44*, 1297–1306.

(23) Hyenstrand, P., Metcalf, J. S., Beattie, K. A., and Codd, G. A. (2001) Losses of the cyanobacterial toxin microcystin-LR from aqueous solution by adsorption during laboratory manipulations. *Toxicon 39*, 589–594.

(24) Ellman, G. L. (1959) Tissue sulfhydryl groups. *Arch. Biochem. Biophys. 82*, 70–77.

(25) Dai, M., Xie, P., Liang, G., Chen, J., and Lei, H. (2008) Simultaneous determination of microcystin-LR and its glutathione conjugate in fish tissue by liquid chromatography-tandem mass spectrometry. *J. Chromatogr. B 862*, 43–50.

(26) Buratti, F. M., and Testai, E. (2007) Evidences for CYP3A4 autoactivation in the desulfuration of dimethoate by the human liver. *Toxicology 241*, 33–46.

(27) Pegram, R. A., Humpage, A. R., Neilan, B. A., Runnegar, M. T., Nichols, T., Thacker, R. W., Pflugmacher, S., Etheridge, S. M., and Love, A. H. (2007) Chapter 15: Cyanotoxins Workgroup Report. In *Cyanobacterial Harmful Algal Blooms: State of the Science and Research Needs* (Hudnell, H. K., Ed.) pp 317−381, Springer, New York; http://www.epa.gov/cyano_habs_symposium/monograph/Ch15.

(28) Satoh, K. (1995) The high non-enzymatic conjugation rates of some glutathione S- transferases (GST) substrates at high glutathione concentrations. *Carcinogenesis 16*, 869–874.

(29) Gibbs, J. P., Czerwinski, M., and Slattery, J. T. (1996) Busulfan-glutathione conjugation catalyzed by human liver cytosolic Glutathione S-Transferases. *Cancer Res. 56*, 3678–3681.

(30) Zhang, K., Wong, K. P., and Chow, P. (2003) Conjugation of chlorambucil with GSH by GST purified from human colon adenocarcinoma cells and its inhibition by plant polyphenols. *Life Sci. 72*, 2629–2640.

(31) Eklund, B., Moberg, M., Bergquist, J., and Mannervik, B. (2006) Divergent activities of human Glutathione Transferases in the bioactivation of azathioprine. *Mol. Pharmacol. 70*, 747–754.

(32) Gibbs, J. P., Yang, J. S., and Slattery, J. T. (1998) Comparison of human liver and small intestinal glutathione *S*-transferase-catalyzed busulfan conjugation *in vitro*. *Drug Metab. Dispos. 26*, 52–55.

(33) Rowe, J. D., Nieves, E., and Listowsky, I. (1997) Subunit diversity and tissue distribution of human glutathione S-transferases: Interpretations based on electrospray ionization-MS and peptide sequence-specific antisera. *Biochem. J. 325*, 481–486.

(34) van Ommen, B., Bogaards, J. J. P., Peters, W. H. M., Blaauboer, B., and van Bladeren, P. J. (1990) Quantification of human hepatic glutathione S-transferases. *Biochem. J. 269*, 609–613.

(35) Coles, B. F., Chen, G., Kadlubar, F. F., and Radominska-Pandya, A. (2002) Interindividual variation and organ-specific patterns of glutathione S-transferase alpha, mu, and pi expression in gastrointestinal tract mucosa of normal individuals. *Arch. Biochem. Biophys. 403*, 270–276.

(36) Zhang, J., Gonzales, V., and Xu, M. J. (2002) Expression and regulation of glutathione S-transferase P1−1 in cultured human epidermal cells. *J. Dermatol. Sc. 30*, 205–214.

(37) Caccuri, A. M., Antonini, G., Ascenzi, P., Nicotra, M., Nuccetelli, M., Mazzetti, A. P., Federici, G., Lo Bello, M., and Ricci, G. (1999) Temperature Adaptation of Glutathione *S*-Transferase P1-1: A case for homotropic regulation of substrate binding. *J. Biol. Chem. 274*, 19276–19280.

(38) Buratti, F. M., Volpe, M. T., Fabrizi, L., Meneguz, A., Vittozzi, L., and Testai, E. (2002) Kinetic parameters of OPT pesticide desulfuration by c-DNA expressed human CYPs. *Environ. Toxicol. Pharmacol. 11*, 181–190.

(39) Buratti, F. M., D'Aniello, A., Volpe, M. T., Meneguz, A., and Testai, E. (2005) Malathion bioactivation in the human liver: The contribution of different cytochrome P450 isoforms. *Drug Metab. Dispos. 33*, 295–302.

(40) Eaton, D. L., and Bammler, T. K. (1999) Concise review of the Glutathione S-transferases and their significance to toxicology. *Toxicol. Sci. 49*, 156–164.

(41) Coles, B. F., and Kadlubar, F. F. (2005) Human alpha class glutathione S-transferases: Genetic polymorphism, expression, and susceptibility to disease. *Methods Enzymol. 401*, 9–42.

(42) Xiong, Q., Xie, P., Li, H., Hao, L., Li, G., Qiu, T., and Liu, Y. (2010) Acute effects of Microcystins exposure on the transcription of antioxidant enzyme genes in three organs (liver, kidney, and testis) of male Wistar rats. *J. Biochem. Mol. Toxicol. 24*, 362–367.

(43) Li, X. Y., Liu, Y. D., Song, L. R., and Liu, J. T. (2003) Responses of antioxidant systems in the hepatocytes of common carp (*Cyprinus carpio*) to the toxicity of microcystin-LR. *Toxicon 42*, 85–89.

(44) Jayaraj, R., Anand, T., and Rao, P. V. L. (2006) Activity and gene expression profile of certain antioxidant enzymes to microcystin-LR induced oxidative stress in mice. *Toxicology 220*, 136–146.

(45) Puerto, M., Pichardo, S., Jos, A., Prieto, A. I., Sevilla, E., Frias, J. E., and Camean, A. M. (2010) Differential oxidative stress responses to pure Microcystin-LR and Microcystin-containing and non-containing cyanobacterial crude extracts on Caco-2 cells. *Toxicon 55*, 514–522.

(46) Soares, R. M., Yuan, M., Servaites, J. C., Delgado, A., Magalhães, V. F., Hilborn, E. D., Charmichael, W. W., and Azevedo, S. M. F. O. (2006) Sub-lethal exposure from microcystins to renal insufficiency patients in Rio de Janeiro - Brazil. *Environ. Toxicol. 21*, 95–103.

(47) Hilborn, E. D., Carmichael, W. W., Soares, R. M., Yuan, M., Servaites, J. C., Barton, H. A., and Azevedo, S. M. F. O. (2007) Serologic evaluation of human microcystin exposure. *Environ. Toxicol. 22*, 459–463.