Calmodulin inhibition as a mode of action of antifungal imidazole pharmaceuticals in non-target organisms

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Abstract

To improve assessment of risks associated with pharmaceutical contamination of the environment, it is crucial to understand effects and mode of action of drugs in non-target species. The evidence is accumulating that species with well-conserved drug targets are prone to be at risk when exposed to pharmaceuticals. An interesting group of pharmaceuticals released into the environment is imidazoles, antifungal agents with inhibition of ergosterol synthesis as a primary mode of action in fungi. However, imidazoles have also been identified as competitive antagonists of calmodulin (CaM), a calcium-binding protein with phylogenetically conserved structure and function. Therefore, imidazoles would act as CaM inhibitors in various organisms, including those with limited capacity to synthesize sterols, such as arthropods. We hypothesized that effects observed in crustaceans exposed to imidazoles are related to the CaM inhibition and CaM-dependent nitric oxide (NO) synthesis. To test this hypothesis, we measured (i) CaM levels and its gene expression, (ii) NO accumulation and (iii) gene expression of NO synthase (NOS1 and NOS2), in the cladoceran Daphnia magna exposed to miconazole, a model imidazole drug. Whereas significantly increased CaM gene expression and its cellular allocation were observed, supporting the hypothesized mode of action, no changes occurred in either NO synthase expression or NO levels in the exposed animals. These findings suggest that CaM inhibition by miconazole leads to protein overexpression that compensates for the loss in the protein activity, with no measurable downstream effects on NO pathways. The inhibition of CaM in D. magna may have implications for effect assessment of exposure to mixtures of imidazoles in aquatic non-target species.

Key words: calmodulin, Daphnia magna, immunostaining, gene expression, nitric oxide synthase, postabdominal organ

Introduction

The increased public awareness of local waterways’ health and concerns about pharmaceutical contamination of surface waters calls for the development of methods for science-based assessment of human impacts in aquatic organisms [1]. Understanding and predicting effects of these newly emerged contaminants and their metabolites are challenging tasks, given a general scarcity of ecotoxicological data for the majority of produced pharmaceuticals [2, 3]; this becomes even more complicated for effect assessment of exposure to mixtures of these compounds [4, 5]. When it comes to assessing the risk of pharmaceuticals for non-target species, the evidence is accumulating that species with...
well-conserved drug targets are more likely to be at risk when exposed to pharmaceutical residues in the environment [6, 7]. In this context, an interesting group of pharmaceuticals released into the environment isazole fungicides (imidazoles and triazoles). The intensive use of imidazole fungicides in medicine and agriculture may potentially lead to substantial environmental contamination [8]. As imidazole derivatives are often used in combination in clinical practice and are present in even more complex mixtures in wastewater effluents, where they are commonly combined with agricultural imidazole fungicides [6], it is crucial to understand their effects on non-target organisms, such as algae and invertebrates.

Azole fungicides are primarily known as antifungal substances interfering with cytochrome P450-dependent (primarily 14α-demethylase, CYP51) fungal synthesis of ergosterol [9, 10]. They are also known to inhibit a range of other cytochrome P450 enzymes [11, 12], such as aromatase (CYP19) [13–15]. In crustaceans, they have shown antecdysteroidal activity [16] and developmental and reproductive toxicity [7, 17], including down-regulating genes related to molting and reproduction [7, 18]. Furthermore, Dalhoff et al. [19] reported that the imidazole prochloraz inhibits CYP450 activity in Daphnia magna. Moreover, this imidazole was found to be a much stronger inhibitor than triazole propiconazole, similar to what has been found for human cytochromes [19]. Although this study does not provide a direct mechanistic evidence for CYP450 inhibition to be the primary pharmacokinetic mechanism behind the observed effects on development and reproduction, it supports the hypothesis that inhibition of cytochrome CYP450 enzymes by azoles could be of significance also in crustaceans.

Toward mechanistic understanding of imidazole ecotoxicity in non-fungal eukaryotes, another pathway deserves a particular attention. It has long been recognized that imidazole derivatives are competitive antagonists of calmodulin (CaM), a calcium-binding protein that has a highly conserved structure in different tissues and species [20]. CaM is a major mediator of Ca²⁺ signaling expressed in all eukaryotic cells and transducing the effects of cytosolic Ca²⁺ signals on cellular processes [21]. It is also involved in the regulation of many channels that generate Ca²⁺ signals. CaM regulates essential protein targets, e.g. CaM-dependent adenylyl cyclases, nitric oxide synthases (NOSs), protein kinases and phosphatases, thereby affecting many different cellular functions, particularly neurotransmitting activity [22]. Therefore, many CaM-dependent cellular functions can be modulated by CaM antagonists, and a common approach in investigating the action of CaM has been to use such membrane-permeant pharmacological antagonists, e.g. calmidazolium [23, 24]. The mechanism by which CaM antagonists inhibit CaM activity involves a direct binding of the drugs to CaM. In multicellular organisms, inhibition of CaM-bridging pathways may have complex downstream effects on behavior, development and growth.

The amino acid sequence of CaM is highly conserved in eukaryotes [25]. Moreover, a very high drug target similarity (~98%) for an ortholog in Daphnia pulex (Crustacea, Cladocera) to the human drug target for CaM regulator was predicted [6]. This conserved nature implies that CaM-inhibiting pharmaceuticals are likely to be also effective in non-target species, and Daphnia can serve as a model test species for these effects. Moreover, in crustaceans, imidazole derivatives are likely to primarily act as CaM inhibitors as arthropods are unable to synthesize sterols and rely on dietary sources for obtaining these compounds [26]. Imidazoles have also been found to inhibit CaM-dependent nitric oxide (NO) synthesis [27], a physiological process important for regulation of the crustacean nervous system and muscle contraction [28]. We therefore hypothesized that exposure to imidazoles inhibit CaM activity and disrupt CaM-dependent NO synthesis in crustaceans. To test this hypothesis, levels of CaM and NO as well as gene expression of CaM and NOS were measured in the cladoceran D. magna exposed to the model imidazole miconazole.

Materials and Methods

Chemicals

Miconazole nitrate was purchased from Sigma Aldrich (purity > 98%). In the experiments below, the nominal concentrations of miconazole were based on the proportion of pure chemical (i.e. 86.85%). Dimethyl sulfoxide (DMSO > 99.5% p.a., Merck) was purchased from VWR International, Stockholm, Sweden.

Test organism

Daphnia magna is a freshwater crustacean and an established model species in ecotoxicological, ecological and evolutionary studies [29–31]. The animals were cultured in M7 medium [32, 33] in groups of ~25 females in 2 L containers and fed a mixture of the green algae Pseudokirchneriella subcapitata and Scenedesmus subspicatus three times a week. All daphniids used in bioassays originated from the same clone (environmental pollution test strain “Klon 5,” the Federal Environment Agency, Berlin, Germany).

Experiments

CaM immunostaining. In vivo immunostaining was used to assess CaM distribution and levels in juvenile daphniids (24–48 h old) exposed to miconazole. Prior to the immunostaining, daphniids were exposed to miconazole (0.2 and 2 mg/L) for 24 h in 20 mL medium without food and a light:dark regime of 16:8 h. Such high concentrations were necessary to allow visual detection of CaM using the whole-body staining. For each test concentration and the solvent control (M7 with 0.1% DMSO, v/v), we used two replicates, five individuals each.

After the exposure, the animals were used for whole-body immunostaining. All immunostaining procedures were conducted at room temperature, and the daphniids were washed twice with phosphate-buffered saline (PBS), pH 7.4, for 10 min between the steps. Daphniids were fixed in methanol for 10 min at room temperature, washed and incubated with chitinase (4 unit/mL in PBS, Sigma) at 30°C for 1 h to digest exoskeleton. The chitinase-treated animals were incubated with rabbit polyclonal anti-CaM antibody (Cell Signaling Technology) diluted 1:500 in PBS; Sigma) at 30°C for 1 h to digest exoskeleton. The chitinase-treated animals were incubated with rabbit polyclonal anti-CaM antibody (Cell Signaling Technology) diluted 1:50 in PBS for 1 h followed by incubation with secondary anti-rabbit DyLight488 conjugated antibody (Thermo Scientific) diluted 1:200 in PBS for 1 h. Then, daphniids were mounted in Mowiol 4–88 medium (Calbiochem) on a microscope slide with a glass cover slip and examined using inverted fluorescent microscope Nikon Eclipse Ti-U equipped with halogen light source and 20× PlanFluor objective. Images were captured with Leica Application Suite software using DFC 420C digital video camera (Leica) and analyzed with ImageJ v. 1.46 software (Image, NIH). The fluorescence intensity was quantified as integral density in the standardized region of interest (ROI) for each specimen and averaged within the group.
Gene expression analysis. Daphniids exposed to 0.05 mg/L miconazole and a corresponding control (7–8 ind./replicate, n = 5) were used to assay differential expression of CaM and two NOS genes (NOS1 and NOS2) by real-time quantitative PCR; β-actin was used as a reference gene. Total RNA was extracted using the RNeasy Mini kit with on-column DNase treatment (Qiagen, UK), following the manufacturer’s instructions. The quantity and quality of RNA were determined on a NanoPhotometer (Implen, Germany). Reverse transcription and amplification were conducted using Power SYBR® Green RNA-to-CT™ 1-Step Kit (Applied Biosystems, Foster, CA, USA) in a 20 μL reaction volume according to the protocol recommended by the manufacturer. Primers from Chen et al. [34] originally developed for the barnacle Balanus amphitrite (forward: 5′-CGTCTGTATTGGTTTCCA-3′ and reverse: 5′-TGACTGACATGTATCCGCGAT-3′) were used to amplify a 116 bp CaM fragment. Using primers from Labbé et al. [35], a 99 bp fragment of NOS1 (forward: 5′-GAGCTCTCCACCCACGGT-3′ and reverse: 5′-AGAGCTCAGCATATCAGC-3′) and a 106 bp fragment of NOS2 (forward: 5′-AGTCCGATTTCATCTCTGC-3′ and reverse: 5′-ACCTCGGTGAAATGGACATT-3′) were amplified. Primers for a 71 bp fragment for β-actin (A)J29554; forward: 5′-CCACACTTGCCTCCCCATTTGAAG-3′ and reverse: 5′-GGCGAACGGCAATCC-3′) were adopted from Kim et al. [36]. The reaction was conducted by StepOne (Applied Biosystems) real-time PCR with reverse transcription. The thermal profile consisted of 30 min of reverse transcription at 48 °C one cycle and 10 min of polymerase activation at 95 °C, followed by 40 cycles of PCR at 95 °C for 15 s and 60 °C for 60 s. Following amplification, a melting curve analysis was performed to verify the authenticity of the amplified product by its specific melting temperature following the instructions of the manufacturer. Validation experiments confirmed that the efficiencies of the target and endogenous control (β-actin) amplifications were similar (94–98%). The comparative threshold cycle method [37] was used to assess the relative levels of target gene mRNAs normalized to those of β-actin.

Statistics

Kruskal–Wallis (KW) statistics and Dunn’s multiple comparison test were used to evaluate differences in (i) integral density in immunostained samples and (ii) NO concentrations among the groups. To determine the deviation of gene expression in D. magna exposed to miconazole in relation to the controls, Student’s t-test was applied. The data were analyzed using GraphPad Prism 6.01 (GraphPad Software Inc.), significance level α = 0.05.

Results

CaM immunostaining

Using whole-body preparations and CaM-specific antibodies, the CaM protein staining was observed in the insertion bulb of setae on both branches of swimming antennae and in the postabdominal organ that is a gravity-sensing organ in cladocerans located at the base of the two abdominal setae in the postabdominal cavity. The most intense fluorescence was observed in postabdominal organ, and therefore, the organ and its surrounding area were defined as ROI (Fig. 1A) in the image analysis. The staining intensity differed significantly between miconazole-treated and control daphnids (KW ANOVA; KW statistic = 9.674, P < 0.008; Fig. 1A and B), whereas no difference was observed between the animals exposed to different miconazole concentrations (Dunn’s multiple comparison test; P > 0.05).

NO measurements. Test solutions were prepared immediately prior to use with culture media M7 and miconazole dissolved in DMSO (0.1%). The exposure experiment consisted of a miconazole treatment (0.05 mg/L miconazole) and a solvent control (DMSO concentration as in the treatment), five replicates per group. Each exposure chamber contained 250 mL of the test solution and 15 juvenile D. magna (instars 2–3); no food was provided. After 6 h exposure, daphnids were rinsed with distilled water, transferred to cryotubes (8 ind./replicate) and stored at −20 °C until the analyses. The remaining individuals were used for gene expression analysis (Section 2.3.3).

To measure NO (nmol/ind.), a non-enzymatic colorimetric assay (Oxford Biomedical Research, Rochester Hills, MI, USA) was used. Animals from the miconazole treatment and solvent control were rinsed and homogenized with acid-washed glass beads (<200 μm, 50 μg) in Mini Beadbeater-8 (Biospec Products, Bartlesville, OK, USA). Homogenates were centrifuged at 10 000 × g for 5 min and analyzed according to the manufacturer instructions. Absorbance was measured using 0.2 mL cuvettes and Hitachi spectrophotometer.

Gene expression analysis. Daphniids exposed to 0.05 mg/L miconazole and a corresponding control (7–8 ind./replicate, 25–75% response ranges (top and bottom lines of boxes) and minima and maxima (whiskers). Non-matching letters denote significant differences (P < 0.05) in the miconazole-exposed specimens when compared to the control. Number of replicates in each group is specified at the top of the figure. Changes in expression are also indicated in terms of fold change relative to the normalized control level.
expression that were upregulated in concert. Using whole body in vivo immunostaining of daphniids exposed to miconazole, we observed the greatest increase in CaM protein levels in the postabdominal organ. It is probable that CaM protein concentrations have increased in all tissues, but were visually detectable only in the postabdominal organ and, to a weaker degree, at the base of swimming setae, most probably, due to relatively high basal CaM expression level in these structures that are intimately involved in sensing and neurotransmitting in cladocerans. Similarly, in situ hybridization in barnacles (Cirripedia) revealed that the expressed CaM gene was localized in structures that have essential functions during larval swimming and settlement, including thorax posterior ganglion [34], which functionally is analogous to the postabdominal organ in Cladocera.

In addition to the increase in CaM concentration, we also observed a significant increase in CaM mRNA synthesis that occurred at 4-fold lower miconazole concentration compared to the effects detected by immunostaining. In line with these observations, imidazoles have been reported to increase CaM concentration by inhibiting its activity [27]. However, they also were shown to inhibit activity of CaM-dependent NOS in vitro [27]. Contrary to the latter and to the hypothesized increase in NO production, no effects on either NO concentration in animal tissue or NOS expression were observed. This suggests that CaM inhibition by miconazole enhances expression of CaM and this overexpression compensates for the loss in the protein activity, with no measurable downstream effects on the NO pathways. Thus, this compensatory response of protein expression appears to be an adaptation to the inhibition of the protein function [38–40].

These findings implicate inhibition of CaM activity as important part of MOA for imidazole effects in daphniids, and, most likely, in other crustaceans. In D. magna, negative effects on development and reproduction by low μg/L-levels of miconazole were related to inhibition of cuticle protein and vitellogenin expression [7]. Given that these proteins are intimately involved in reproduction and development and that calcium-binding CaM controls many physiological functions, including ecdysteroid production in crustaceans [41], it is possible that these responses are related to the inhibition of CaM activity induced by miconazole. Potential disruption of the molting cycle in crustaceans has also been linked to the Ca$^{2+}$-CaM-mediated activation of NOS and release of NO, which increase intracellular cyclic guanosine monophosphate that, in turn, leads to suppression of ecdysteroidogenesis [42]. In larval barnacles, Ca$^{2+}$ -CaM-dependent phosphodiesterase inhibitors suppressed larval attachment and metamorphosis in a dose-dependent manner [43]. In line with this, more recent studies suggest a complex involvement of CaM and its binding proteins to the development and metamorphosis of invertebrates [34, 44]. Also other azole fungicide classes have been linked to disturbances in ecdysteroidogenesis and molting in crustaceans. For instance, fenarimol that belongs to the pyrimidine class of demethylase inhibitors exhibited antiecdysteroidal activity in D. magna by lowering endogenous ecdysone levels and delaying molting in a concentration-dependent fashion [16]. Later, Soetaert et al. [18] showed that this compound exerts a major action on molting-specific pathways at the gene expression level by down-regulating several cuticle proteins in D. magna. These studies provide strong evidence that azoles, being both CaM and CYP inhibitors, have negative effects on larval development and competency in crustaceans. However, there is scarce information in the scientific literature whether there may exist links between CYP and CaM activation/inhibition. Alvarez et al. [45] proposed

**Figure 2**: NO concentrations (nmol/ind.; median value, 25–75% response ranges, minima and maxima) in D. magna exposed to miconazole (0.05 mg/L) and the controls; n = 5

**Figure 3**: Box plots of mRNA expression for CaM, NOS1 and NOS2 relative to the normal control state after normalization to β-actin. Shown are the median value (horizontal line), 25–75% response ranges (top and bottom lines of boxes) and minima and maxima (whiskers). Asterisks indicate a significant change (horizontal line), 25–75% response ranges (top and bottom lines of boxes) and minima and maxima (whiskers). Asterisks indicate a significant change

NO concentrations

There was no treatment effect on either mean NO values (t-test; t8 = 0.0738, P > 0.9) or the variance (F test; F4,4 = 4.839, P > 0.2; Fig. 2) in the D. magna exposed to miconazole.

Gene expression

A significant increase was observed for CaM expression in daphniids exposed to miconazole in relation to the control animals (t10 = 3.818, P < 0.004; Fig. 3), whereas no significant treatment effects were observed for either NOS1 or NOS2 (NOS1: t10 = 0.8192, P > 0.432; NOS2: t10 = 0.3983, P > 0.678; Fig. 3).

Discussion

Our results support the hypothesized mode of action (MOA) of imidazoles in relation to their toxicity in non-target organisms. The inhibition of CaM activity was likely the drug’s action in D. magna as indicated by the protein levels and its gene
that activation of a CYP P450 situated at intracellular Ca²⁺ stores in rat thymocytes could open a Ca²⁺ pathway that in turn would be inhibited by Ca²⁺ inside the cells by a CaM-dependent mechanism. Whether similar pathways involving both CYP and CaM are present in crustaceans remains to be investigated.

Ägerstrand et al. [46] have recommended that the cumulative risk for groups of pharmaceuticals with similar MOA should be assessed to increase the relevance of the environmental risk assessment process of pharmaceuticals. Considering the intensive use of imidazoles in both medicine and agriculture and the risk for substantial environmental contamination [8], our findings that inhibition of CaM activity is a likely MOA in non-target crustaceans could therefore be of significant importance in the effect assessment of exposure to mixtures of these substances.

**Conflict of interest statement**

No potential conflict of interest was reported by the authors.

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**References**

1. Kümerer K. The presence of pharmaceuticals in the environment due to human use - present knowledge and future challenges. J Environ Manage 2009;90:2354–66.
2. Fent K, Weston AA, Caminada D. Ecotoxicology of human pharmaceuticals. Aquat Toxicol 2006;76:122–59.
3. Molander I, Ägerstrand M, Rudén C. WikiPharma – a freely available, easy accessible, interactive and comprehensive database for environmental effect data for pharmaceuticals. Regul Toxicol Pharmacol 2009;55:367–71.
4. Eggen RIL, Behr R, Burkhardt-Holm P et al. Challenges in ecotoxicology. Environ Sci Technol 2004;38:58A–64A.
5. Quinn B, Gagné F, Blaise C. Evaluation of the acute, chronic and teratogenic effects of a mixture of eleven Pharmaceuticals with evolutionary conserved molecular drug targets in organisms? J Environ Manage 2009;407:1072–9.
6. Gunnarsson L, Jauhiainen A, Kristiansson E et al. Environmental antiecdysteroids alter embryo development in the crustacean Daphnia magna. J Exp Zool 2002;292:287–92.
7. Hassold E, Backhaus T. Chronic toxicity of five structurally diverse demethylase-inhibiting fungicides to the crustacean Daphnia magna: a comparative assessment. Environ Toxicol Chem 2009;28:1218–26.
8. Soetaert A, van der Ven K, Moens LN et al. Daphnia magna and ecotoxicogenomics: gene expression profiles of the anti-ecdysteroidal fungicide fenarimol using energy-, molting- and life stage-related cDNA libraries. Chemosphere 2007;67:60–71.
9. Dalhoff K, Gottardi M, Kretschmann A et al. What causes the difference in synergistic potentials of propiconazole and prochloraz toward pyrethroids in Daphnia magna? Aquat Toxicol 2010;172:95–102.
10. Hegemann L, Tojo SM, Laihijani KI et al. Direct interaction of antifungal azole-derivatives with calmodulin: a possible mechanism for their therapeutic activity. J Invest Dermatol 1993;100:343–6.
11. Friedberg F, Rhoads AR. Evolutionary aspects of calmodulin. IUBMB Life 2001;51:215–21.
12. Shin D, Means AR. Calmodulin: a prototypical calcium sensor. Trends Cell Biol 2000;10:322–8.
13. Sunagawa M, Kosugi T, Nakamura M et al. Sterol biosynthesis and metabolism in marine invertebrates. Pure Appl Chem 1981;53:837–52.
14. Wolff DJ, Datto GA, Samatovicz RA. The dual mode of inhibition of calmodulin-dependent nitric-oxide synthase by antifungal imidazole agents. J Biol Chem 1993;268:9340–6.
15. Kim H-W, Batista LA, Hoppes JL et al. A crustacean nitric oxide synthase expressed in nerve ganglia, Y-organ, gill and gonad of the tropical land crab, Gecarcinus lateralis. J Exp Biol 2007;207:2845–587.
16. Shaw JR, Frenner M, Eads BD et al. Daphnia as an emerging model for toxicological genomics. Adv Exp Biol 2008;2:165–219.
17. Van der Ven K, Moens LN, Van den Bossche H et al. The water flea Daphnia - a 'new' model system for ecology and evolution? J Biol 2010;9:21.
32. OECD (Organization for Economic Cooperation and Development). *Daphnia magna* reproduction test. OECD Guidelines for Testing of Chemicals. Technical Guidance Document 211, 1998.

33. OECD (Organization for Economic Cooperation and Development). *Daphnia* sp. acute immobilisation test. OECD Guidelines for Testing of Chemicals. Technical Guidance Document 202, 2004.

34. Chen ZF, Wang H, Matsumura K et al. Expression of calmodulin and myosin light chain kinase during larval settlement of the barnacle *Balanus amphitrite*. *PLoS One* 2012;7:e31337.

35. Labbé P, McTaggart SJ, Little TJ. An ancient immunity gene duplication in *Daphnia magna*: RNA expression and sequence analysis of two nitric oxide synthase genes. Dev Comp Immunol 2009;33:1000–10.

36. Kim J, Kim Y, Lee S et al. Determination of mRNA expression of DMRT93B, vitellogenin, and cuticle 12 in *Daphnia magna* and their biomarker potential for endocrine disruption. *Ecotoxicology* 2011;20:1741–8.

37. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 2001;25:402–8.

38. Miller MJ, Thompson JH, Liu X et al. Failure of L-NAME to cause inhibition of nitric oxide synthesis: role of inducible nitric oxide synthase. *Inflamm Res* 1996;45:272–6.

39. Skolness SY, Durhan EJ, Garcia-Reyero N et al. Effects of a short-term exposure to the fungicide prochloraz on endocrine function and gene expression in female fathead minnows (*Pimephales promelas*). *Aquat Toxicol* 2011;103:170–8.

40. Balasubramanian S, Kanade S, Han B et al. A proteasome inhibitor-stimulated Nrf1 protein-dependent compensatory increase in proteasome subunit gene expression reduces polycomb group protein level. *J Biol Chem* 2012;287:36179–89.

41. Mattson MP, Spaziani E. Regulation of crab Y-organs steroidogenesis in vitro - evidence that ecdysteroid production increases through activation of cAMP-phosphodiesterase by calcium-calmodulin. Mol Cell Endocrinol 1986;48:135–51.

42. Nakatsuji T, Lee CY, Watson RD. Crustacean molt-inhibiting hormone: structure, function, and cellular mode of action. Comp Biochem Physiol Part A Mol Integr Physiol 2009;152:139–48.

43. Yamamoto H, Tachibana A, Saikawa W et al. Effects of calmodulin inhibitors on cyprid larvae of the barnacle, *Balanus amphitrite*. *J Exp Zool* 1998;280:8–17.

44. Luan J, Geng JP, Fang SQ et al. Developmental expression pattern of calmodulin gene in amphioxus *Branchiostoma belcheri tsingtauense*. *Chinese J Oceanol Limnol* 2010;28:1221–6.

45. Alvarez J, Montero M, Garciasonchasro J. Cytochrome-P450 may regulate plasma-membrane Ca$^{2+}$ permeability according to the filling state of the intracellular Ca$^{2+}$ stores. *FASEB J* 1992;6:786–92.

46. Ågerstrand M, Berg C, Björlenius B et al. Improving environmental risk assessment of human pharmaceuticals. *Environ Sci Technol* 2015;49:5336–45.