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Influence of P-glycoprotein on embryotoxicity of the antifouling biocides to sea urchin (*Strongylocentrotus intermedius*)

Xue Xu · Jingxuan Fu · Heng Wang · Baidong Zhang · Xia Wang · Yonghua Wang

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Abstract P-glycoprotein (P-gp), as an ATP-binding cassette transporter, transports a wide variety of substrates varying from small molecules like steroids to large polypeptides across the cell membrane in human and animals, even in aquatic animals. Although P-gp protein has attracted much attention of research, its effect on the toxicity of environmental toxicants such as antifouling biocides is still poorly understood. The goal of this study is to evaluate whether copper pyrithione (CuPT), Sea-Nine 211, dichlofluanid and tolylfluanid, four widely used antifouling agents, can be transported by P-gp in embryos of sea urchin *Strongylocentrotus intermedius* in the presence and absence of the P-gp inhibitor verapamil. Cytotoxcicities of Sea-Nine 211 (EC50 = 99 nM, at 4-arm pluteus) and dichlofluanid (EC50 = 144 nM, at multi-cell) are enhanced by the addition of the P-gp inhibitor, indicating that the two biocides are potential P-gp substrates. Tolylfluanid and CuPT are not transported by P-gp out of the cell, since no obvious changes in the cytotoxicities of the two biocides are observed no matter whether verapamil is added or not. In addition, to understand the mechanisms of ligand binding and its interaction with P-gp, a three-dimensional model of the sea urchin P-gp is generated based on the mouse crystal structure by using homology modeling approach. With this model, a flexible docking is performed and the results indicate that Sea-Nine 211 and dichlofluanid share the same binding site with verapamil, composed of key residues Lys677, Lys753, Thr756, Ala780, Met1033 and Phe1037, whereas tolylfluanid and CuPT display totally different binding modes to P-gp. This further demonstrates that Sea-Nine 211 and dichlofluanid are P-gp substrates, which provides us with new insights into the interactions of P-gp with the antifouling contaminants in aquatic invertebrate embryos.

Keywords P-glycoprotein · Antifouling biocides · Sea urchin embryotoxicity, docking · Homology modeling

X. Xu · H. Wang · B. Zhang · X. Wang · Y. Wang (✉)
College of Life Sciences, Northwest A&F University, Yangling 712100, Shaanxi, China
e-mail: yh_wang@nwsuaf.edu.cn

J. Fu
Key Laboratory of Mariculture & Biotechnology, Ministry of Agriculture, Dalian Ocean University, Dalian 116023, Liaoning, China

Y. Wang
Center of Bioinformatics, Northwest A&F University, Yangling 712100, Shaanxi, China

Introduction

The ATP-binding cassette (ABC) transporters represent a superfamily of more than 100 membrane transporters/channels that are responsible for a multiplicity of functions, including the extrusion of harmful compounds, uptake of nutrients, transport of ions and peptides, and cell signaling (Leslie et al. 2005). For most ABC transporters, the binding and subsequent hydrolysis of ATP at their nucleotide binding domains are essential for providing energy for the movement of their substrates across membranes (Leslie et al. 2005, Higgins 2007). As one of the typical ABC transporters, P-glycoprotein (P-gp) is a large, glycosylated membrane protein which localizes predominantly to the plasma membrane of the cell (Van Tellingen 2001). Besides its characteristics associated with multidrug resistance, it confers the protection of cells against harmful substrates by active, ATP-dependent extrusion of a range of cytotoxic drugs from the cell (Van Tellingen 2001;

Balayssaca et al. 2005; Wang et al. 2005). Structurally, it is composed of two bundles of six transmembrane helices (TMs 1–3, 6, 10, 11 and TMs 4, 5, 7–9, 12), resulting in an inward-facing conformation. The arrangement of the "two halves" forms a large internal cavity open to both the cytoplasm and the inner leaflet that is cubed with a separation of the two nucleotide-binding domains (Aller et al. 2009).

In recent years, expression of the P-gp transporter has been correlated with cell protection against xenobiotics and environmental pollutants in many different species of insects and marine organisms, and P-gp appears to play a critical role in environmental toxicology (Porretta et al. 2008; Venn et al. 2009; Bošnjak et al. 2010; Sun et al. 2009; Xu et al. 2010a, b). In the mosquito *Aedes caspius*, toxicity of two commonly-used insecticides temephos and diflubenzuron has been shown to increase by inhibition of P-gp, suggesting the potential involvement of P-gp in insecticide defense (Porretta et al. 2008). The interactions between pesticides with P-gp have also been investigated on reef corals, showing that in vitro exposition of copper sulphate and Corexit 9527 could induce the expression of P-gp in the reef corals (Venn et al. 2009). Recently, the presence of P-gp has been reported in sea urchin *Strongylocentrotus purpuratus*, showing that expression of such genes protected the embryos of sea urchin from toxic xenobiotic compounds (Hamdouna et al. 2004). And the transport function of P-gp in sea urchin has been further demonstrated in the fertilization studies of *Paracentrotus lividus* and *Arbacia lixula* that revealed the protective role of the multidrug resistance-associated (MRP) protein-mediated multi-xenobiotic defense (Bošnjak et al. 2010). Our previous work has also suggested that the early sea urchin embryos of *Strongylocentrotus intermedius* expressed P-gp which was associated with tolerance to pesticides cyromazine and ethiofencarb (Sun et al. 2009).

As environmental monitors, the embryo–larval bioassays, in particular with bivalves and sea urchins, have been used for decades as sensitive, simple, and reliable tools for assessing and monitoring marine pollution (Bellas 2007; Xu et al. 2010a, b). Despite much work, little information has been published about the influence of P-gp on the toxicity of environmental contaminants, especially antifouling biocides, to sea urchin embryos at different developmental stages. Such information is urgently required in order to evaluate the environmental risk of these compounds in view of reported high concentrations in coastal environment.

Antifoulants, which prevent the settlement and growth of marine organisms on submerged structures, are recognized universally (Kevin et al. 2003). In the last decade, as alternatives for TBT-based paints, 18 "TBT-free" compounds including copper pyrithione, dichlofluanid, tolylfluanid and Sea-Nine 211 have been introduced and applied as a new generation of antifouling biocides, and approved for use by Health and Safety Executive (HSE) in amateur and professional antifouling products marketed in the UK (HMSO 1998; Voulvoulis et al. 2002). However, accompanied with the frequent application of these biocides in many countries such as UK (Gatidou et al. 2007), Japan (Hiroya et al. 2007) and China (Kueh and Lam 2008), high coastal concentrations have been found in areas of high yachting activity, particularly in marinas and sportive harbors. Even though great concern has been taken into account with the prescription of environmental risk assessment of biocides in recent years, several questions still remain to be clarified: (1) Whether the contamination in the aquatic environment caused by the alternatives has toxic effect on marine species living in the water, and (2) since organisms have self-protective mechanisms against the noxious effects of xenobiotics such as the ABC system, whether this immune system is self-sufficient at affording protection against exogenous toxicants for the marine organisms.

Therefore in the present study, we investigated the effects of P-gp on the toxicity of four antifouling biocides (copper pyrithione, dichlofluanid, tolylfluanid and Sea-Nine 211), using the model of sea urchin embryos *Strongylocentrotus intermedius* (*S. intermedius*) at different developmental stages (2-cell, 4-cell, 8-cell, multi-cell, blastula, swimming blastula, gastrula, prism, and 4-arm pluteus). Firstly, an in vitro model (embryos of sea urchin) allowed us to estimate the toxic effect of single antifouling biocide to the embryos of *S. intermedius*. Then the embryos were treated with the biocides in conjunction with a typical P-gp inhibitor verapamil. We focused on the comparison of the individual toxicity with the joint toxic effects to determine which biocides can be transported by P-gp. Moreover, to study the interaction mechanism of the antifouling biocides with the P-gp protein at atomic level, we built a three-dimensional (3D) structure of P-gp by homology modeling technique that was subsequently used to explore the binding modes of the antifoulants with the P-gp by molecular docking.

Materials and methods

Biological material

Experiments were performed from November to December, 2009. Mature *S. intermedius* (diameter = 6.0 ± 0.5 cm, approximately 2 years old) were fed in flow-through filtered natural seawater (FSW) (0.45 μm filter) system at 13 ± 1°C.

In the present study, the gametes of sea urchin were harvested with embryos reared according to the standard

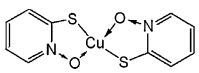
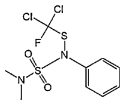
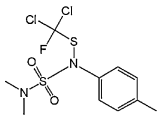
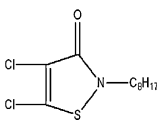
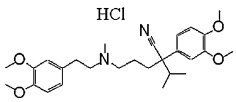
protocol described by Pagano et al. (1986). At the beginning of each assay the sea urchins were induced to spawn using 0.5 M KCl (3.73 g of KCl in 100 ml of distilled water) (1–2 ml). The quality of the eggs and sperm was previously assessed and the sperm to egg ratio was calibrated to optimize fertilization. Then eggs suspension (100–120 eggs/ml) and the sperm solution were stored at 20 and 4°C until use, respectively.

Test chemicals

The stock solutions were made up by dissolving the analytical grade antifoulants and the P-gp inhibitor in FSW approximately 1 h before the beginning of the experiments. The chemicals were CuPT (bis-(hydroxy-2(H)-pyridine thionate-O,S)-copper), dichlofluanid (1,1-dichloro-*N*-[(dimethylamino)sulfonyl]-1-fluoro-*N*-phenylmethanesulfenamide), tolylfluanid (1,1-dichloro-*N*-[(dimethylamino)sulfonyl]-1-fluoro-*N*-(4-methylphenyl)methanesulfenamide), Sea-Nine 211 (4,5-dichloro-2-n-octyl-4-isothiazolin-3-one) and verapamil hydrochloride (a-[3-[[2-(3,4-Dimethoxyphenyl)ethyl] methylamino]propyl]-3,4-dimethoxy-a-(1-methylethyl)benzeneacetonitrile hydrochloride) obtained from Sigma-Aldrich, Steinheim, Germany unless otherwise specified. The properties including the structures of these compounds are shown in Table 1.

The test media contained 1% non-toxic dimethylsulfoxide (DMSO) that was required to pre-dissolve CuPT and Sea-Nine 211, and 1% acetone for dichlofluanid and tolylfluanid. Physicochemical conditions of the experiments were 31.19 ± 0.45 ppt salinity, 7.09 ± 0.22 mg/L dissolved O_2, and 8.0 ± 0.05 pH (mean + S.D., $n = 6$) during the whole test.

Table 1 Structural information of four antifouling biocides and verapamil

Chemical[a]	CAS No.	Structure
Copper pyrithione	14915-37-8	
Dichlofluanid	1085-98-9	
Tolylfluanid	731-27-1	
Sea-Nine 211	64359-81-5	
Verapamil hydrochloride	152-11-4	

[a] All the chemicals are obtained from Sigma-Aldrich, Steinheim, Germany

Toxicity effects

The toxic effects of the pharmacological treatment were determined by the embryonic development of *S. intermedius* in liquid medium (Boiocchi and Toffoli 1992). The solutions of these compounds were diluted to get final six concentrations respectively, for CuPT with the experimental concentrations from 0.05 to 911.25 nM, for dichlofluanid (from 50 to 25,600 nM), for tolylfluanid (from 5 to 91,125 nM) and for Sea-Nine 211 (from 6 to 3200 nM), and the vials were incubated at 20°C. The exposure time of each embryo sample to toxicants was 50 h from fertilization. Non-exposed embryos were used as controls.

Sea urchin is known to exhibit radial holoblastic cleavage (http://www.stanford.edu/group/Urchin/contents.html), and in this study, we observed the embryo development at stages of 2-cell, 4-cell, 8-cell, multi-cell, blastula, swimming blastula, gastrula, prism, and 4-arm pluteus, respectively. In the first seven embryo cleaves, the cells divided meridionally, and finally, produced a 128-cell blastula in the seventh division. During this period, the stages of 2-cell, 4-cell, 8-cell, and multi-cell were observed at 2, 3, 4 and 6 h, respectively. In the blastula stage of sea urchin, however, cells were very different from the first seven cleavages, which formed a hollow sphere surrounding a central cavity at 11 h. At this time, embryos initially exhibited differentiation. As the cells continued to divide, they secreted a hatching enzyme that digested the fertilization envelope at the swimming blastula stage at 19 h. During gastrulation, the germ layers of embryos were further formed at 26 h and the body plan of the mature organism was established. The prism stage at 41 h was characterized by a change in the overall shape of the embryo (a rounded, truncated pyramid). When the preoral and the postoral arms were completely formed, the larval developed to the 4-arm pluteus stage at 50 h (Wang et al. 2010; Xu et al. 2010a, b).

Drug toxicity was calculated as the percentage of embryo survival in the drug-treated cultures compared with that in the untreated controls. In the experiments with verapamil (750 nM) (which did not affect embryo development), the embryos treated with verapamil alone were used as controls (Eepl et al. 2006). The results were finally reported as median 50% effective concentration (EC50) values for the chemical toxicity to the embryos of *S. intermedius*.

Homology modelling

Due to unavailability of the X-ray structure of sea urchin P-gp, a homology modeling for the protein structure from its primary sequence was performed (Thompson et al. 1994). The template protein employed here was mouse P-gp (PDB code: 3G5U chain A (Aller et al. 2009)) which exhibited a high resolution (3.0 Å). The target protein was ABCB1, whose amino acid sequence (ID: NP_001029122.1) was taken from the NCBI Web site (http://www.ncbi.nlm.nih.gov).

The initial sequence alignment of the target and the template sequences was carried out using the ClustalW program (Thompson et al. 1994). The homology structure models of the putative sea urchin P-gp were then generated using the program SWISS-MODEL (Automated Comparative Protein Modeling Server, Version 3.5, Glaxo Wellcome Experiment Research, Geneva, Switzerland) (http://swissmodel.expasy.org). Final versions of the models were subsequently submitted to the protein structure verification WHAT-CHECK module of the WHATIF on-line server (http://swift.cmbi.ru.nl/gv/whatcheck/) for validation. All H-atoms were finally added to the unoccupied valence of heavy atoms at the corresponding neutral state using the biopolymer module of SYBYL 6.9 package (http://www.tripos.com/sybyl).

Molecular docking

Docking, playing a critical role in the rational design of agents, is frequently used to predict the binding orientation of drug candidates to their protein targets (active sites) and also to predict the binding affinity of the molecules in turn (Jain 2003). In this study, in order to illustrate the binding modes of the active sites of P-gp with the antifoulants, docking was carried out using the Surflex module of SYBYL package. Firstly, structures of the ligands were constructed with standard bond lengths and angles and their energies were minimized using the Powel method with a conjugated gradient of <0.05 kcal/mol convergent criteria provided by the Tripos force field and electrostatic charges based on the method of Gasteiger-Hückel. Secondly, the Surflex employed sea urchin P-gp as an identified active site to generate putative poses of molecules or molecular fragments. Before docking, all water molecules and the originally cocrystallized ligands were removed and hydrogen atoms were added to the protein. The appropriate conformations for these compounds were determined by leave-one-out cross-validation procedure, ensuring the total scores derived from the Surflex were well correlated with their observed biological activities.

Statistical analyses

Embryogenesis success reached high values in the control (95%). The tests of the four antifoulants on the biological responses were repeated six times ($n = 6$) and the data were expressed as mean ± standard deviation (S.D.). The EC50 values defined here, and their 95% confidence intervals (95% CI) were calculated according to the Bliss probit analysis method (Bliss 1935). Differences were considered to be statistically significant when $P \leq 0.05$ by comparing the experimental groups with the control groups.

Results and discussion

Antifouling paints have been widely used to prevent and control the growth of unwanted organisms on the surfaces of artificial structures immersed in the sea (Townsin 2003). However, some of them can cause deleterious ecotoxicological effects, mainly due to their environmental stability and toxicity to non-target organisms such as imposex and decreased reproductive viability in gastropods, or increased shell thickness in oysters, which becomes an increasing worldwide concern (Alzieu 2000; Evans et al. 2000).

With the development of alternative biofouling biocides, researchers are not only interested in the toxic effects of biocides in the aquatic environments, but also focus emphasis on how to prevent the emerging environmental problems. Since P-gp is a typical transport protein, this gives rise to the idea that this type of protein might play an important role in the defense of the body against xenobiotics by excreting these compounds out of the cells. Unfortunately, even though the interaction of pesticides with P-gp has already been investigated (Shabbir et al. 2005; Venn et al. 2009), the protective effect of P-gp on the toxicity of antifouling biocides to the sea urchin embryos in the coastal waters is still poorly characterized. Therefore, in this work, we focus on the toxicities of antifoulants on the sea urchin embryos, particularly to uncover whether P-gp limits the bioavailability of the biocides and contributes to their excretion out of cells.

Single toxicity

The single effects of four antifoulants on the EC50 values of *S. intermedius* are shown in Fig. 1a. For the first division before the 4-cell stage, the toxicity of chemicals to sea urchin early development embryos is as follows: dichlofluanid > tolylfluanid > Sea-Nine 211 > CuPT, and their corresponding 1-octanol/water partition coefficient (log *P*)

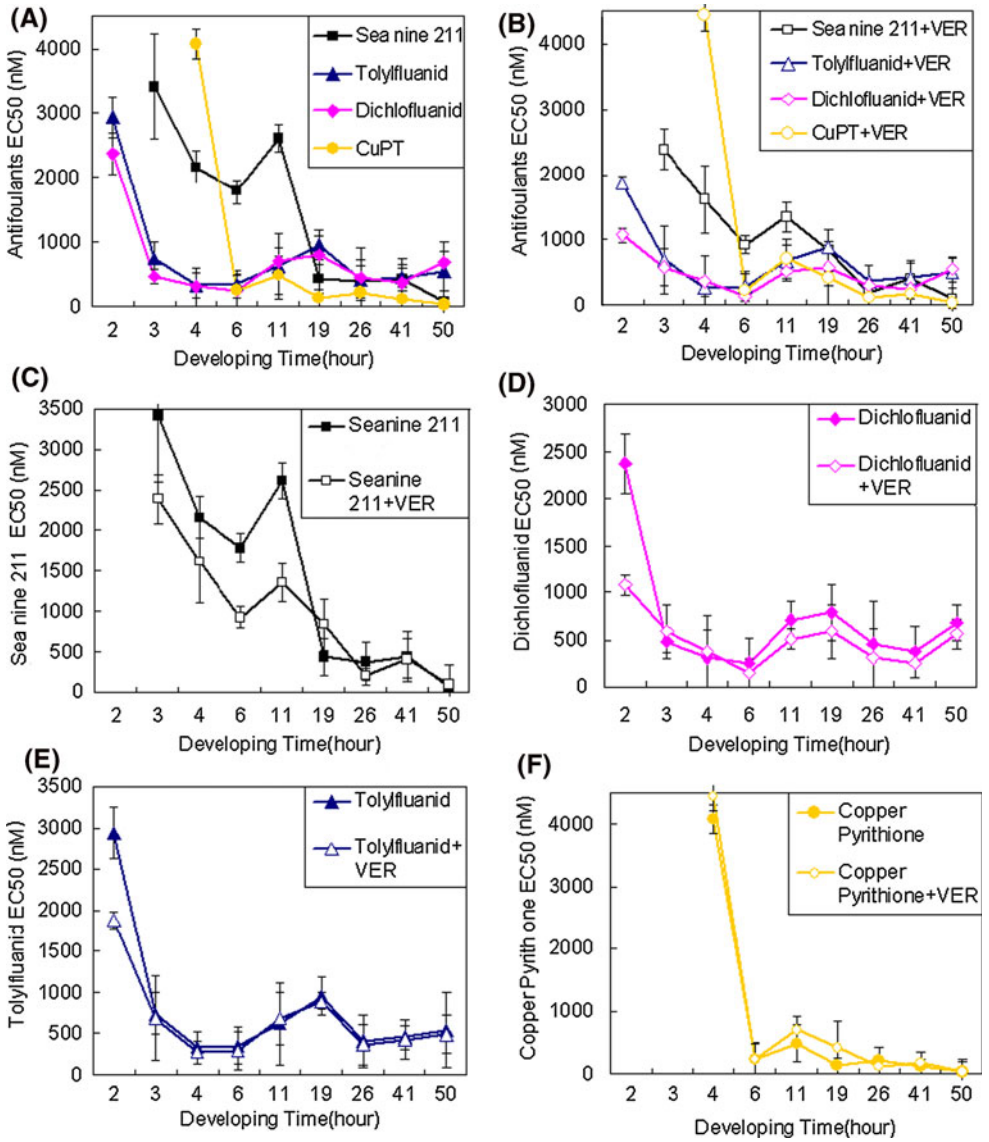


Fig. 1 **a** Single toxic effects of Sea-Nine 211 (*solid square*), tolylfluanid (*solid triangle*), dichlofluanid (*solid diamond*), and copper pyrithione (*solid circle*) to the embryos of sea urchin *S. intermedius* at 2-cell, 4-cell, 8-cell, multi-cell, blastula, swimming blastula, gastrula, prism, and 4-arm pluteus, respectively. The toxicity is quantified in terms of the EC50 (median effective concentration) reducing embryogenesis success by 50%. **b** Joint toxicity of the Sea-Nine 211 + verapamil mixture (*open square*), the tolylfluanid + verapamil mixture (*open triangle*), the dichlofluanid + verapamil mixture (*open diamond*), and the copper pyrithione + verapamil mixture (*open circle*) at different developmental stages, respectively. **c** A comparison of the single toxicity of Sea-Nine 211 with the joint toxicity of the Sea-Nine 211 + verapamil mixture at different developmental stages. **d** A comparison of the single toxicity of dichlofluanid with the joint toxicity of the dichlofluanid + verapamil mixture at different developmental stages. **e** A comparison of the single toxicity of tolylfluanid with the joint toxicity of the tolylfluanid + verapamil mixture at different developmental stages. **f** A comparison of the single toxicity of copper pyrithione with the joint toxicity of the copper pyrithione + verapamil mixture at different developmental stages. Data are obtained from at least six parallel experiments. *Error bars* represent ± standard deviation ($n = 6$)

values are 3.7, 3.9, 2.8 and 4.0, respectively. Our previous work confirmed that the embryos of sea urchin are more sensitive to highly hydrophobic compounds since these chemicals preferentially permeate the lipid bilayers (hydrophobic compartments) of cells (Xu et al. 2010a, b). In this study, the toxicity of the three chemicals dichlofluanid, tolylfluanid and Sea-Nine 211, within the experimental error, supports the log *P*-dependent rule, but that of CuPT does not. This indicates that the toxicity level of CuPT is not only associated with its hydrophobic properties, but probably also linked with its much lower solubility (0.1 mg/l (Sandel et al. 2003)) compared with the other three compounds, hence hindering its membrane-permeation and resulting in the low toxicity (4,000 nM at

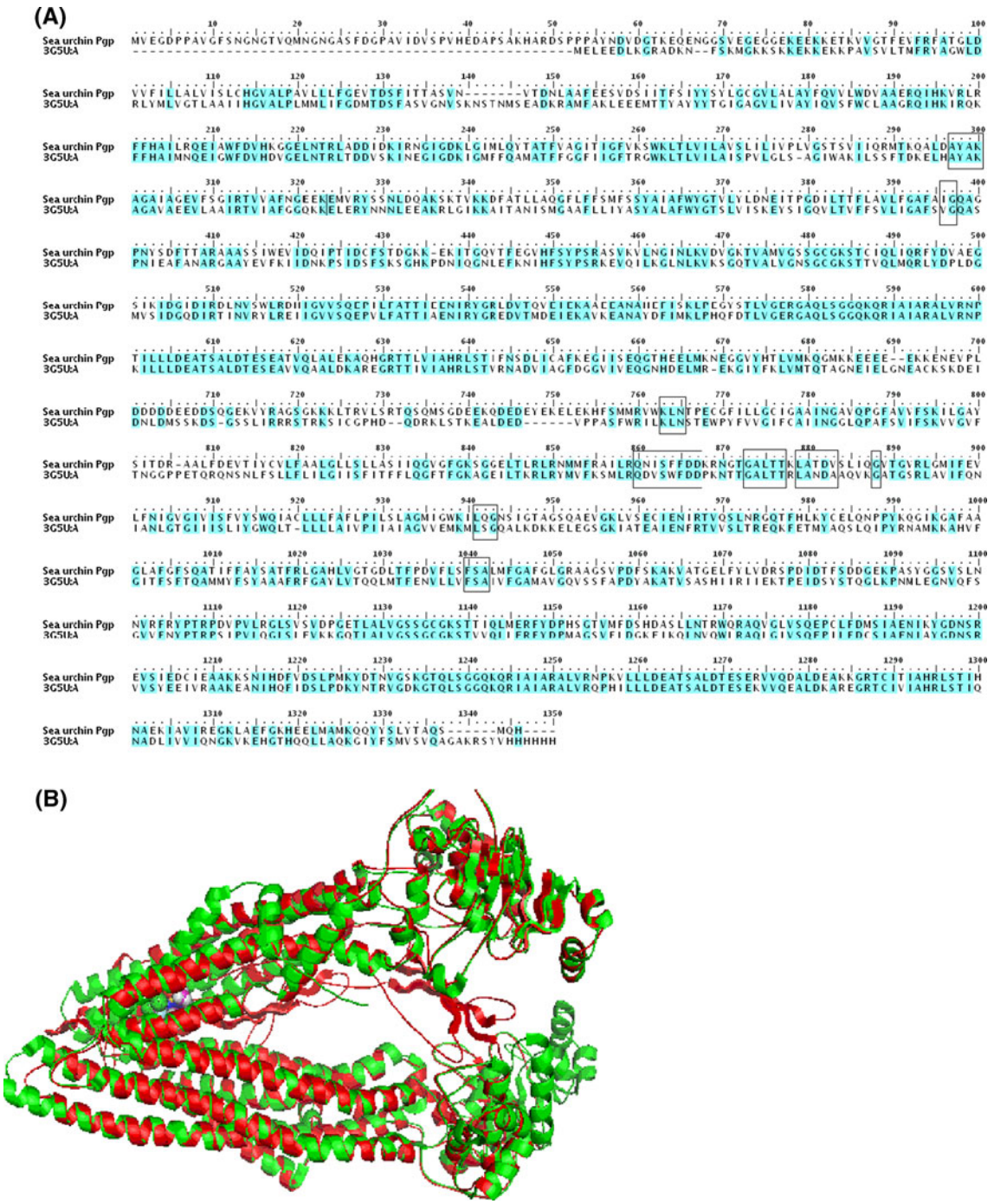


Fig. 2 **a** Alignments of the sequences of 3G5U chain A template with the target sea urchin P-gp. *Shadow regions* denote that the residues in the individual column are identical in the sequence alignment. *Dashed lines* denote the deletion of amino acids. The binding sites of the four antifoulants and verapamil are highlighted in *rectangles*. **b** Superposition of the 3D-structure of 3G5U chain A template (*green ribbon*) with the sea urchin P-gp model (*red ribbon*) obtained from homology modeling (Color figure online)

2-cell). Further, we observe that following a sharp decrease in the EC50 value of CuPT from 4,000 to 45 nM at 4-cell, the toxicity of this compound maintains at a relatively lower level (about 40 nM) in other developmental stages. This result indicates that once CuPT permeates through the membranes of sea urchin embryos, it could induce significantly toxic effects on the cells. In the costal water, the lower solubility of CuPT increases its effective availability as a biocidal agent over a longer period of time when exposed to marine environments (Koutsaftis and Aoyama 2007), but also, makes it the most toxic agent to the marine organisms among the four biocides.

For compounds dichlofluanid, tolylfluanid and Sea-Nine 211, however, their toxicity gradually decreases with the development of the embryos of *S. intermedius* as indicated by the curves in Fig. 1a, and all of them show significantly similar toxic effects ((EC50 = ~70 nM) from the swimming blastula stage to the 4-arm pluteus stage. During these *S. intermedius* developmental stages, the cells of embryos are very different from the first four divisions. They differentiate to increase morphological heterogeneity through the arrangement of cells into increasingly complex structural patterns in the form of tissues and organs, such as the three germ layers (ectoderm, mesoderm, and endoderm) during gastrulation, and the ophagus, stomach, and intestine regions at the prism stage (http://www.stanford.edu/group/Urchin/contents.html). Just due to the embryonic differentiation, the embryos of *S. intermedius* form a complex system of tissues and cell types. Under such condition, even though the toxicants successfully permeate through the membranes, they could not distribute evenly among all organs in the larval of sea urchin, therefore possibly leading to the less sensitivity of the embryos to the environmental perturbations.

Inhibition analysis

Many compounds are known to modulate P-gp by reducing the efflux activity of the pump, such as aldosterone, doxorubicin and verapamil (Ford and Hait 1990). As a particularly important inhibitor used in studying P-gp, verapamil shows the greatest ability to inhibit the ATPase activity of P-gp (Loo and Clarke 2001). In this study, to determine whether the presence of verapamil alters the toxic effects of the four antifoulants, this chemical is introduced into the test solutions (Whalen et al. 2010; Sun et al. 2009), and kept at a concentration of 750 nM in each mixture in all tests, thus assuring its nontoxic effect to the embryos but effective inhibition on the P-gp-mediated drug transport (Epel et al. 2006).

In the case of treatment with verapamil, no obvious change is seen in the testes of embryos treated with tolylfluanid + verapamil, and CuPT + verapamil (Fig. 1e, f), while in the treatment with Sea-Nine 211 + verapamil, and dichlofluanid + verapamil, the addition of verapamil significantly increases the toxicity of Sea-Nine 211 (threefold) and dichlofluanid (twofold) as almost no overlap is observed between the EC50 values of biocide alone and biocide + verapamil (Fig. 1c, d).

As shown in Fig 1e, verapamil almost does not inhibit the P-gp-mediated transport of tolylfluanid except at the 2-cell stage; at such stage, the joint toxicity (tolylfluanid + verapamil) increases from 2,800 nM (EC50) to 1,873 nM (EC50), more than onefold higher than the single toxicity of tolylfluanid. This suggests a low potential for tolylfluanid to cause drug interactions at the P-gp level, and a stage-specific expression of P-gp. Similarly, the combination of CuPT and verapamil also does not cause significant increase in the toxicity of CuPT. However, at 8-cell and multi-cell, we observe moderately enhanced toxic effects (about 100 nM) of the mixture (Fig. 1f). Since the toxic response curves for the single and mixture tests are well overlapped on each other at the remaining stages, we propose that CuPT could not be transported by the P-gp pump.

Interestingly, when verapamil is added to the Sea-Nine 211-related culture plates, the toxicity of Sea-Nine 211 is stimulated about threefold in the first four divisions before the blastula stage, increasing by about 1,000 nM (Fig. 1c). This indicates the effectively inhibitory effects of verapamil on the P-gp-mediated drug transport, and the involvement of P-gp in defense against Sea-Nine 211. However, at other developmental stages, no obvious change is observed between the joint and the single toxicity. This draws us a conclusion that following with the development of the embryos of *S. intermedius*, the inhibitory effect of verapamil might become weaker, which cannot inhibit the P-gp-based transport, consequently resulting in the slight difference between the joint and the single toxicity from the blastula to the 4-arm pluteus stages.

For the combination of dichlofluanid and verapamil, the toxicity shows a large increase at the 2-cell stage (EC50 = 1,075 nM), which is about twofold higher than the single toxicity (EC50 = 2,440 nM) (Fig. 1d). Also, the mixture exhibits strengthened toxicity from the blastular stage compared with the individual toxicity (about 150 nM), which is not observed in the toxicity of other mixtures. This implies that verapamil has a dramatic effect on the inhibition of P-gp transport, and also, P-gp, as a drug efflux pump, can efficiently modify the intracellular accumulation of dichlofluanid and hence the cytotoxicity.

The above data thus reflect two interesting findings: (1) P-gp genes exhibit stage-specific expression and show pump functions at 3 h after fertilization, which is consistent with our previous studies (Sun et al. 2009). And (2) Sea-Nine 211 and dichlofluanid are potential P-gp substrates, because once P-gp is inhibited by verapamil, the toxicity of the two compounds significantly increased by 150–1000 nM.

Homology modeling and molecular docking analysis

As described above, the protein P-gp can, to some extent, affect the acute toxicity of the antifouling biocides (CuPT, Sea-Nine 211, tolylfluanid and dichlofluanid) to the embryogenesis of *S. intermedius*, respectively. However, the detailed mechanism of how P-gp interacts with the antifouling agents at an atomic level remains unclear. To predict the binding modes of the biocides with P-gp, the

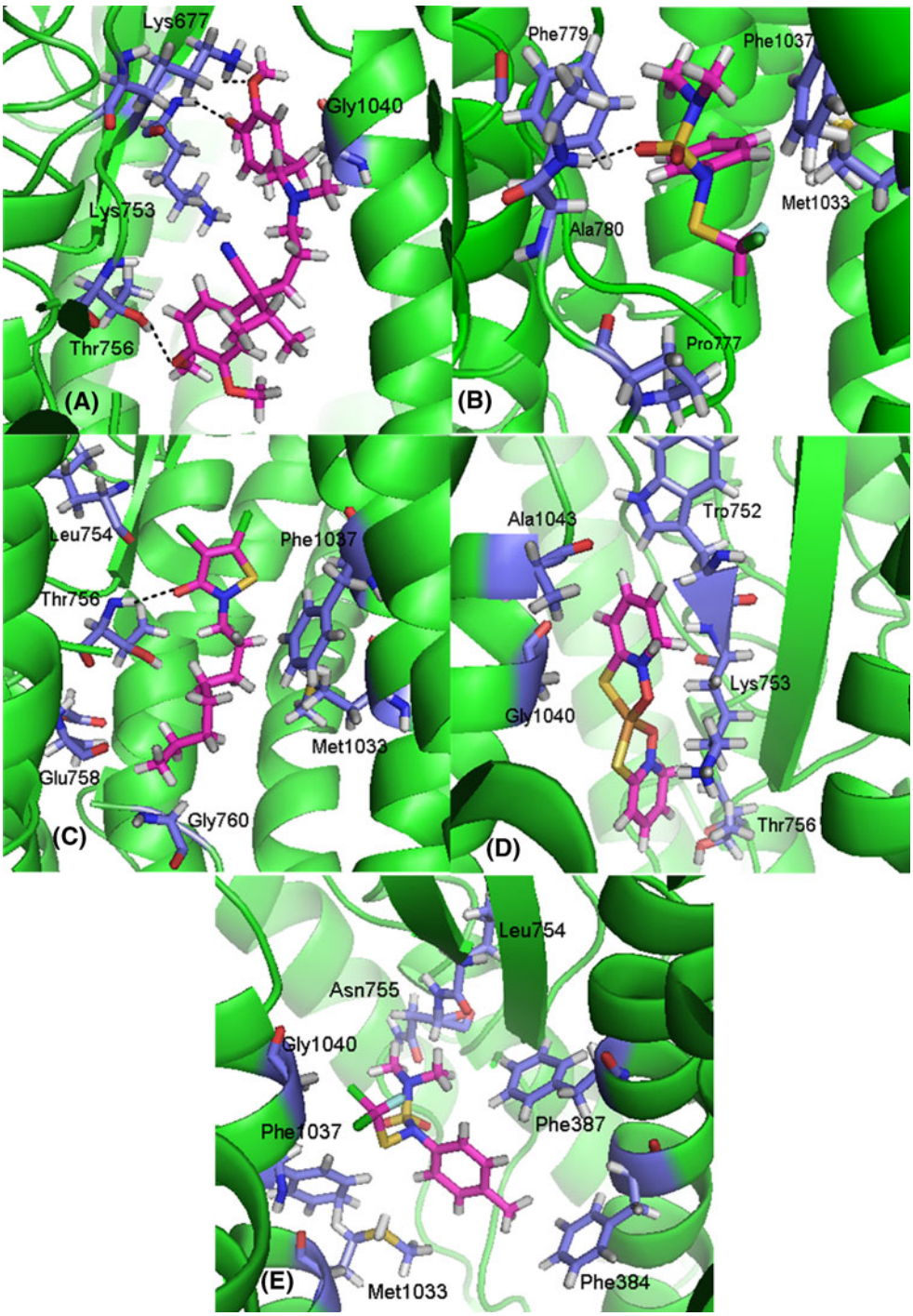


Fig. 3 Close-up views of the binding modes of **a** verapamil, **b** dichlofluanid, **c** Sea-Nine 211, **d** copper pyrithione and **e** tolylfluanid. The binding sites of P-gp are shown as ribbons, with their neighboring residues in stick representation. The five small molecules are also indicated in stick form. The *dotted lines* are the hydrogen bonds (H-bonds) between the molecules and their neighboring residues

molecular docking thus has been performed based on the homology model of sea urchin P-gp.

The 3D structure of sea urchin P-gp is modeled by using the SWISS-MODEL workspace at the ExPASy server with all default parameters. In Fig. 2a, the primary sequence alignment of amino acids shows that the target (sea urchin P-gp) and the reference (mouse P-gp) proteins share about 51% sequence identity with E value of 0.0. Particularly, the residues in the P-gp ligand-binding channel reveal a sequence identity of 83% between the template and the target proteins, indicating that these function-related amino acids are well conserved in the two organisms. As expected, superposition of the 3D homology with the template models also shows a high similarity in the relative abundances of α-helix motif and the atomic positions (Fig. 2b). All these, therefore, indicate the homology-modeled P-gp structure is reliable and can be used for further docking analysis.

As depicted in Fig. 3a, the small molecule verapamil is well located in the center of the P-gp active site, and forms three hydrogen bonds (H-bond) with its neighboring residues (2.03 Å with Lys753, 1.95 Å with Lys677 and 2.92 Å with Thr756). The interactions significantly enhance the stability of the verapamil-P-gp complex with the highest total score of 7.7 among the five molecules, which is consistent with previous experimental findings, showing that the protein P-gp could strongly interact with verapamil (Yusa and Tsuruo 1989).

By comparison of the location of verapamil in P-gp with those of dichlofluanid and Sea-Nine 211, we find an interesting phenomenon that the three molecules occupy the same binding site of P-gp. For the dichlofluanid-P-gp system, dichlofluanid is surrounded by residues Ala780, Pro777, Phe779, Met1033 and Phe1037. And just one H-bond is observed between its sulfuryl oxygen with the amino group of Ala780, thus resulting in a much lower binding affinity of 2.93 with P-gp. However, for Sea-Nine 211, this molecule interacts strongly with its adjacent residues (Leu754, Thr756, Glu758, Gly760, Phe1037 and Met1033), even forming the same H-bond with Thr756 (2.12 Å) as verapamil does, and hence, presents a relatively high binding affinity (total score = 4.2). On the basis of the above theoretical analysis, we suggest that verapamil competitively inhibits the interactions between the two biocides with the P-gp drug binding site, therefore inhibiting the drug-transporting function of P-gp and enhancing the toxicity of the biocides to the embryos of *S. intermedius* (Fig. 1c, d). This is consistent with previous studies, showing that most P-gp inhibitors are supposed to work by blocking the P-gp substrate binding sites (Varma et al. 2003; Aller et al. 2009), or interacting with the same binding site of P-gp and sharing many common structural features with other substrates (Wang et al. 2005).

As for molecules tolylfluanid and CuPT, their highest docking scores are 1.82 and 3.02, respectively, implying the weak-binding interactions with P-gp (Fig. 3d, e). This could be explained by further analysis that no strong interactions such as H-bonds or ion bonds are found in the two ligand–protein complexes, and the two biocides even do not have the same binding site as verapamil in P-gp. Thus, consistent with our experimental result in this work, CuPT and tolylfluanid might not be the substrates of P-gp, and meanwhile, P-gp could not transport them from the cytoplasmic leaflet to the extracellular aqueous medium (Fig. 1e, f).

Conclusions

As a membrane-spanning protein, P-gp has been shown providing a defense against the environmental xenobiotics. To examine the possible role of P-gp in defence against the antifouling biocides in *S. intermedius*, we conducted bioassays using four biocides (CuPT, Sea-Nine 211, tolylfluanid and dichlofluanid) alone and biocides plus verapamil, an inhibitor of P-gp. Our results suggest that Sea-Nine 211 and dichlofluanid are P-gp substrates and their toxic effects could be profoundly affected by verapamil. However, CuPT and tolylfluanid are not potential P-gp substrates since verapamil has no significant impacts on the two compounds. In addition, the theoretical investigations using the homology modeling technique and molecular docking have provided further support to our experimental observations, indicating that both Sea-Nine 211 and dichlofluanid have the same binding sites as verapamil has, which implies the potential binding modes of the biocides with P-gp. Clearly, these findings are greatly meaningful for environmental risk assessment, and considerably expand our knowledge of P-gp substrates from the antifouling paints-resulted contaminants.

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