



The role of multixenobiotic transporters in predatory marine molluscs as counter-defense mechanisms against dietary allelochemicals

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ABSTRACT

Multixenobiotic transporters have been extensively studied for their ability to modulate the disposition and toxicity of pharmacological agents, yet their influence in regulating the levels of dietary toxins within marine consumers has only recently been explored. This study presents functional and molecular evidence for multixenobiotic transporter-mediated efflux activity and expression in the generalist gastropod *Cyphoma gibbosum*, and the specialist nudibranch *Tritonia hamnerorum*, obligate predators of chemically defended gorgonian corals. Immunochemical analysis revealed that proteins with homology to permeability glycoprotein (P-gp) were highly expressed in *T. hamnerorum* whole animal homogenates and localized to the apical tips of the gut epithelium, a location consistent with a role in protection against ingested prey toxins. *In vivo* dye assays with specific inhibitors of efflux transporters demonstrated the activity of P-gp and multidrug resistance-associated protein (MRP) families of ABC transporters in *T. hamnerorum*. In addition, we identified eight partial cDNA sequences encoding two ABCB and two ABCC proteins from each molluscan species. Digestive gland transcripts of *C. gibbosum* MRP-1, which have homology to vertebrate glutathione-conjugate transporters, were constitutively expressed regardless of gorgonian diet. This constitutive expression may reflect the ubiquitous presence of high affinity substrates for *C. gibbosum* glutathione transferases in gorgonian tissues likely necessitating export by MRPs. Our results suggest that differences in multixenobiotic transporter expression patterns and activity in molluscan predators may stem from the divergent foraging strategies of each consumer.

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1. Introduction

Soft-bodied benthic organisms produce a vast number of structurally diverse secondary metabolites, many of which function as feeding deterrents in marine systems (Hay and Fenical, 1988; Harvell and Fenical, 1989; Paul, 1992; Hay, 1996; Stachowicz, 2001). For marine consumers, the consequences of ingesting chemically defended prey can be quite severe (Targett and Arnold, 2001), yet specialized consumers that solely feed on toxic prey can apparently tolerate these dietary compounds, and in some cases, even concentrate the defensive compounds for their own protection (Cimino et al., 1985; Cronin, 2001). Few studies have explored the physiological targets of these compounds in generalist consumers or the mechan-

isms by which more specialized predators are able to cope with their toxic prey (Sotka et al., 2009).

The activity of multixenobiotic resistance transporters (MXRs) provides one mechanism by which consumers protect themselves from toxin-laden prey (Epel, 1998). MXR proteins may actively export allelochemicals out of cells or facilitate the sequestration of toxins within specialized cells or organelles, effectively compartmentalizing them away from vulnerable protein and DNA targets (Sorensen and Dearing, 2006). Many of the proteins involved in the transport of xenobiotics across membranes belong to the ATP Binding Cassette (ABC) family. Several members of the ABCB (P-glycoprotein; P-gp or MDR) and ABCC (multidrug resistance-associated protein or MRP) subfamilies function as highly promiscuous transporters, capable of trafficking a diverse array of moderately hydrophobic xenobiotics across cell membranes (Bodo et al., 2003). Together, the over-expression of both P-gp and MRP in tumor cells has long been known to mediate the ATP-dependent efflux of anticancer agents, conferring resistance to natural product chemotherapeutic compounds (Deeley and Cole, 2006; Sarkadi et al., 2006). Only recently has it been suggested that ABC transporters are responsible for regulating the absorption of allelochemicals in the guts of consumers, and may therefore have a significant influence on the foraging patterns and

Abbreviations: ABC, ATP Binding Cassette; C-AM, Calcein-AM; MRP, Multidrug resistance-associated protein; P-gp, P-glycoprotein.

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ultimately diet choice of these organisms (Sorensen and Dearing, 2006; Sotka & Whalen, 2008).

The ubiquity of MXRs in aquatic organisms has been confirmed by immunological cross-reactivity studies, *in vivo* functional assays, competitive inhibition substrate-binding assays (Bard, 2000; Eufemia and Epel, 2000; Scherer et al., 2008; Lüders et al., 2009), and more recently by molecular evidence (Goldstone et al., 2006; Goldstone, 2008; Sturm et al., 2009; Venn et al., 2009). The distribution of MXRs in tissues involved in absorption, secretion and barrier functions in aquatic invertebrates (Bard, 2000) suggests that they may play a role in the prevention of dietary xenobiotic absorption. Furthermore, several pharmacological-based studies have also identified marine natural products from algae, sponges, tunicates, sea hares, gorgonians and marine bacteria that may be substrates for MXR proteins (Suganuma et al., 1988; Chambers et al., 1993; Williams and Jacobs, 1993; Aherne et al., 1996; Quesada et al., 1996; Schroder et al., 1998; Tanaka et al., 2002; Shi et al., 2007; Tanabe et al., 2007; Barthomeuf et al., 2008; Henrich et al., 2009), suggesting that the pool of potential substrates in marine ecosystems may be extensive. Given the myriad allelochemically-rich prey and hosts in marine communities, the constitutive or inducible expression of MXRs may serve as a protective counter-response in marine consumers by reducing dietary toxin absorption.

Studies from both human pharmacology (Marchetti et al., 2007) and aquatic systems (Contardo-Jara et al., 2008; Amé et al., 2009) reveal that natural products from both terrestrial and marine sources can induce the expression and activity of MXRs. If inducers of MXR activity are present in sufficient concentration in the diet of a consumer, ingestion of compounds could result in the enhanced efflux of co-ingested allelochemicals and possibly promote feeding. However, recent evidence also suggests that the unpalatability of some diets may be linked, in part, to the presence of potent MXR inhibitors (Smital et al., 2004) that are produced by the host/prey to directly interfere in efflux activity. These inhibitory compounds could act as “potency enhancers” by blocking transport activity, therefore resulting in increased accumulation of additional noxious allelochemicals (Sorensen and Dearing, 2006). This inhibitory strategy may be employed by chemically defended invasive species to thwart their consumption (Smital et al., 1996; Schroder et al., 1998; Smital et al., 2004) by naïve consumers who may lack the adequate molecular architecture to cope with the invasive’s chemistry. These findings emphasize the need to explore whether marine consumers that are regularly exposed to a diversity of toxic allelochemicals in their diet may have evolved greater tolerance of chemical defenses if they maintain high levels of ABC transporter protein and/or activity in vulnerable tissues.

The objective of this study was to characterize the MXR proteins potentially involved in resistance to dietary allelochemicals in two species of tropical gastropods that feed exclusively on allelochemically defended gorgonian corals. A combination of molecular, immunological and functional approaches were used to examine the expression and activity of molluscan ABC transporters in *Tritonia hamnerorum*, a specialist nudibranch that feeds on a single genus of gorgonian, and *Cyphoma gibbosum*, a generalist gastropod that includes multiple gorgonian families in its diet. Evidence from chemical ecology studies in terrestrial systems suggests that generalists, as a result of their chemically diverse diets, have evolved a greater diversity of catalytically versatile xenobiotic resistance mechanisms as compared to specialists that are exposed to a reduced spectrum of allelochemicals due to their narrow foraging range (Li et al., 2004; Sorensen and Dearing, 2006; Whalen et al., 2010). This study presents the initial characterization of MXRs likely responsible for xenobiotic resistance in these two molluscs as part of an effort to obtain a more complete understanding of how generalists and specialists cope with their allelochemical diet(s) (Whalen et al., in preparation; Whalen et al., 2010).

2. Material and methods

2.1. Animal collection

In 2004, over 200 adult *Tritonia hamnerorum*, ranging in size from 3 mm to 13 mm, were collected from shallow reefs (<10 m) (Big Point – 23°47.383’N, 76°8.113’W; North Normans – 23°47.383’N, 76°8.264’W) surrounding the Perry Institute of Marine Science (PIMS), Lee Stocking Island, Exuma Cays, Bahamas. The purple sea fan, *Gorgonia ventalina*, was the only species of octocoral observed to serve as host for *T. hamnerorum* at our study site. The density of *T. hamnerorum* on individual sea fans ranged from two to over 500 individuals per sea fan. Nudibranchs were collected by removing the portion of sea fan housing them with scissors and transporting both the gorgonian and nudibranchs back to wet laboratory facilities provided by PIMS where they were maintained in flowing filtered seawater until further use. Nudibranchs used for RNA and protein isolation were removed from their host gorgonian, pooled, flash frozen in liquid nitrogen and kept at –80 °C until processing.

In 2006, a total of 141 adult *Cyphoma gibbosum* (ca 2–3 cm length) were collected from five shallow reefs (<20 m) (Big Point – 23°47.383’N, 76°8.113’W; North Normans – 23°47.383’N, 76°8.264’W; Rainbow Gardens – 23°47.792’N, 76°8.787’W; Shark Rock – 23°45.075’N, 76°7.475’W; Sugar Blue Holes – 23°41.910’N, 76°0.23’W) surrounding PIMS. Snails were immediately transported to wet laboratory facilities provided by PIMS, where a series of feeding assays were conducted with seven gorgonian species (*Briareum asbestinum*, *Eunicea mammosa*, *Gorgonia ventalina*, *Pseudopterogorgia acerosa*, *Pseudopterogorgia americana*, *Pseudopterogorgia elisabethae*, *Plexaura homomalla*) observed to serve as hosts for *C. gibbosum* in the field. A detailed description of the feeding assay is reported in Whalen (2008).

2.2. RNA isolation and RT-PCR cloning

Total RNA was isolated from a pool of whole *T. hamnerorum* (267.9 mg; *n*~40 individuals) using the RNeasy Maxi Kit (Qiagen, Valencia, CA) following the manufacturer’s instructions. In addition, a series of feeding assays conducted in 2004 at PIMS with 15 adult *C. gibbosum* feeding on four gorgonian species (*Briareum asbestinum*, *Gorgonia ventalina*, *Pseudopterogorgia acerosa*, *Pseudopterogorgia americana*) provided the material for the initial cloning of ABC transporter cDNA fragments. Upon completion of these feeding assays, *C. gibbosum* digestive glands were immediately dissected and stored in RNALater® at –80 °C (*n* = 15 digestive glands) until further processing. Total RNA was isolated from the pooled *C. gibbosum* digestive glands using RNA STAT-60 (Tel-Test B, Inc., Friendswood, TX, USA) according to the manufacturer’s protocol. Poly(A)+ RNA from both molluscan species was then purified using the MicroPoly(A)Purist mRNA purification kit (Ambion, Austin, TX, USA) according to the manufacturer’s instructions. First-strand cDNA was reverse transcribed from 2 µg poly(A)+ RNA using OmniScript reverse transcriptase (OmniScript RT kit, Qiagen) with random hexamer primers.

Degenerate primers for MRP were a generous gift from David Epel and Amro Hamdoun, Hopkins Marine Station and were designed against the conserved Walker A/B domains (Allikmets and Dean, 1998; Dean et al., 2001) (Supplementary Table S1). PCR and nested PCR were performed using AmpliTaq Gold DNA polymerase (Applied Biosystems) under the following conditions: 94 °C for 10 min; 35 cycles of 94 °C for 15 s, 52 °C for 30 s; 72 °C for 7 min and with degenerate primers pairs (MRP_F/MRP_R and nestMRP_F/nestMRP_R). PCR products were visualized on agarose gels, gel purified (Gene Clean II, Bio 101, Inc.), ligated into pGEM-T Easy® plasmid vector (Promega, Madison, WI, USA), and transformed into JM109 cells (Promega). PCR products were sequenced in both directions using an ABI 3730XL capillary sequencer at the Keck Facility located at the Josephine Bay Paul Center for Comparative Molecular Biology and Evolution at the Marine Biological

Laboratory (Woods Hole, MA, USA). A minimum of twelve clones were sequenced for each PCR fragment. Clones were grouped based on nucleotide sequence with Sequencher 4.6 (Gene Codes Corporation) and a consensus sequence was generated and then examined by NCBI/GenBank BLASTX for gene identification (Altschul et al., 1997). BLASTX searches identified two partial sequences from *C. gibbosum* and two partial sequences from *T. hamnerorum* as having homology to MRP transporters; these were designated as CgMRP isoform 1, CgMRP isoform 2, ThMRP isoform 1 and ThMRP isoform 2.

2.3. Rapid amplification of cDNA ends (RACE)

Initial P-gp cDNA fragments were amplified from both gastropod species by RACE using degenerate primers designed to the nucleotide binding domain (Supplementary Table S1) and adaptor-ligated ds cDNA libraries that were constructed as described below (see Supplemental File S1 for additional information). BLASTX searches identified two partial sequences from *C. gibbosum* and two partial sequences from *T. hamnerorum* as having homology to P-gp transporters; these were designated as CgPgp isoform 1, CgPgp isoform 2, ThPgp isoform 1 and ThPgp isoform 2.

In order to obtain additional 5' and 3' sequence for molluscan ABC transporter cDNAs, gene-specific primers were designed to initial cDNA fragments for RACE. One microgram of poly(A)+ RNA was isolated for both molluscan species as described above and used to create an adaptor-ligated double-stranded cDNA library synthesized using the Marathon cDNA Amplification Kit (BD Biosciences, Palo Alto, CA, USA) according to the manufacturer's instructions. All primers were obtained from Sigma Genosys (St. Louis, MO, USA) and PCR amplification conditions are described in Supplemental File S1. PCR products obtained from *C. gibbosum* and *T. hamnerorum* RACE reactions were ligated into pGEM-T Easy© plasmid vector (Promega, Madison, WI, USA), transformed into JM109 cells (Promega), sequenced in both directions using an ABI 3730XL capillary sequencer at the Keck Facility located at the Josephine Bay Paul Center for Comparative Molecular Biology and Evolution at the Marine Biological Laboratory (Woods Hole, MA, USA), and analyzed as described below.

2.4. Sequence alignments and phylogenetic analysis

Clones were initially grouped based on nucleotide sequence identity (>80%) with Sequencher 4.6 and a consensus sequence was generated and examined by NCBI/GenBank BLASTX for gene identification (Altschul et al., 1997). To place the ABC transporter sequences from *Cyphoma* and *Tritonia* within a phylogenetic context, we pursued two lines of phylogenetic approaches. We first assessed the relative position of gastropod sequences in relation to the full suite of human ABC transporters (i.e., subfamilies ABCA–ABCG). Multiple alignments of molluscan deduced amino acid sequences were performed using ClustalW (Thompson et al., 1994). Human ABC gene subfamilies were aligned using Muscle v3.7 (Edgar, 2004). In order to maintain the proper relationships between the incompletely sequenced ABC domains, *C. gibbosum* and *T. hamnerorum* P-gp and MRP sequences were aligned to each other separately and then to the human ABCB (containing P-gp) and ABCC (containing MRP) families using the profile–profile alignment settings of Muscle. Alignments were automatically masked using the alignment quality scores of Muscle (cut-off score of 20). Maximum likelihood (ML) phylogenetic analyses were performed with RAxML (v7.0.0; Stamatakis, 2006). Two types of analyses were performed: default rapid hill-climbing ML searches, starting from multiple different randomized maximum parsimony (MP) trees; or 100 replicates of rapid MP bootstrapping to find optimal start trees followed by rapid hill-climbing ML optimization. The PROT MIX WAG model of amino acid substitution was used in all analyses; it uses a category model of the WAG amino acid substitution matrix (Whelan and Goldman, 2001) during the hill-climbing ML

search but a gamma distribution of substitution rates for a final tree optimization (Stamatakis, 2006).

In the second approach, we assessed the ABCB and ABCC phylogeny from gastropods and several other invertebrates (the nematode *Caenorhabditis elegans*, the arthropod *Drosophila melanogaster*, the bivalves *Brachidontes pharaonis* and *Mytilus californianus*) and *Homo sapiens*. The putative nucleotide binding domains (hereafter Domains One and Two) were determined using the Conserved Domain Database algorithm on GenBank (Marchler-Bauer et al., 2007). The library SEQINR (Charif and Lobry, 2007) within R (<http://www.r-project.org>) was used to download amino acid sequences from ProtSwiss (<http://www.uniprot.org>) and extract the approximately 215–220 amino acids that constitute each domain. Domains were aligned independently using ClustalX and then alignments were concatenated by hand. The most appropriate model of protein evolution as determined using ProtTest 2.4 (Abascal et al., 2005; based on AIC scores) was LG+I+G (Le and Gascuel, 2008), but because this recently described model is not implemented in most phylogenetic programs, we used the next most fit model (WAG+I+G) (Whelan and Goldman, 2001). A maximum likelihood phylogeny was generated using PHYML v2.4.4 (Guindon and Gascuel, 2003) and supported with 1000 bootstrap replicates. A Bayesian phylogeny was generated using MrBayes (Ronquist & Huelsenbeck, 2003) with two independent runs of 2×10^7 generations each (sampled every 1000 generations) and a burn-in of 20,000 generations. In both ML and Bayesian analyses, the gamma distribution of substitution was estimated using four categories ($\alpha = 2.0$), and the proportion of invariant sites was 0.2. Phylogenies were visualized using FigTree (<http://tree.bio.ed.ac.uk/software/figtree/>) and manipulated using Adobe Illustrator®.

2.5. Real-time quantitative RT-PCR and statistical analysis

MRP isoform 1 transcript expression levels in *C. gibbosum* digestive glands were quantified by real-time quantitative PCR using the iCycler MyiQ Real-Time PCR Detection System (Bio-Rad). Digestive gland total RNA from *C. gibbosum* participating in the 2006 feeding assays was purified and DNase-treated using the RNeasy Maxi Kit and RNase-free DNase Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. Poly(A)+ RNA was isolated using the MicroPoly(A) Purist mRNA purification kit according to the manufacturer's instructions. DNase-treated poly(A)+ RNA (0.2 µg) isolated was used to synthesize cDNA using a blend of oligo (dT) and random hexamers as described by the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Quantitative RT-PCR reactions contained 12.5 µL 2× SYBR Green Supermix reagent (Bio-Rad), 10 ng cDNA, and 100 nM of each gene-specific primer (136_1_F1/136_1_R1, Supplementary Table S1) in a final volume of 25 µL. The PCR conditions were as follows: 95 °C for 3 min; 40 cycles of 95 °C for 15 s, 64 °C for 1 min. PCR product specificity from each primer pair was confirmed by melt curve analysis to ensure that only a single product was amplified. Initially, a representative group of ten poly(A)+ RNA samples was used to perform control cDNA synthesis experiments without reverse transcriptase to check for contamination by genomic DNA. For each RNA sample, relative transcript abundance was calculated from a standard curve that was generated for each qPCR primer set by serially diluting plasmids containing the fragment to be amplified. Each sample and standard was run in duplicate and the expression of *C. gibbosum* β-actin was used to control for differences in cDNA synthesis among samples.

To examine whether *Cyphoma* MRP isoform 1 transcript expression differed between snails feeding on a control diet versus a gorgonian diet, a two-way analysis of variance (ANOVA) was used with Diet (control vs. gorgonian diet) as a fixed factor and Reef (snail origin) as a random factor. The MRP-1 gene was considered a dependent variable. In addition, a one-way ANOVA was used to

examine the variability in MRP-1 expression among reefs, where Reef was considered a random factor. This test was used to investigate reef-specific variation in transcript levels in time-zero snails, and to determine if any such variation persisted in snails collected from these same reefs after being fed a control diet for 4 days. *P*-values were corrected for the two-way ANOVA analysis using by Bonferroni adjustment (Sankoh et al., 1997). Data analysis was performed using SYSTAT® version 11 (Systat Software, Inc., San Jose, CA, USA).

2.6. Western blot for ABC transporters

Crude homogenates were prepared by homogenizing two *C. gibbosum* digestive glands and whole *T. hamnerorum* in 10 mM potassium chloride, 1.5 mM magnesium chloride, 10 mM Tris-HCl, (pH 7.4), containing 2 mM PMSF and 5% (by vol.) SDS at 4 °C. In addition, livers from *Fundulus heteroclitus* collected from New Bedford Harbor, MA, were also homogenized as described and used as a positive control (Bard et al., 2002). The crude homogenate was centrifuged at 14,000 *g* for 2 min at 4 °C, and the supernatant was stored at –80 °C until analysis. Protein concentrations were determined using the bicinchoninic acid (BCA) assay (Smith et al., 1985). Crude tissue homogenates were then diluted to 20 µg total protein with standard SDS-PAGE sample buffer (50 mM Tris, pH 6.8, 2% SDS, 10% sucrose, 0.2% β-mercaptoethanol, 0.001% bromphenol blue), denatured at 100 °C for 3 min, and loaded onto a 7.5% Tris-glycine gel using a 4% stacking gel. Prestained molecular markers were purchased from Amersham (Full-range Rainbow MW Markers, GE Healthcare, Buckinghamshire, UK). Following electrophoresis, proteins were transferred onto a 0.45 µm nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA) at 15 V for 54 min. The membrane was blocked in 5% (wt/vol) non-fat milk in Tris buffered saline-Tween 0.1% (TBST) for 1 h and then hybridized overnight with rocking at 4 °C in TBST containing 0.4 µg/mL of the monoclonal antibody C219 (Signet Laboratories, Dedham, MA, USA). The membrane was subsequently washed (3 × 10 min) with TBST and incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Inc., West Grove, PA, USA) diluted 1:7000 in TBST. Detection of HRP activity was by enhanced chemoluminescence (ECL) onto Hyperfilm ECL (Amersham).

2.7. Immunohistochemical analysis

Live nudibranchs and snails were transferred to a 50 mL beaker containing filtered seawater and were relaxed by adding a few crystals of magnesium sulfate until they no longer recoiled to the touch. Digestive glands and mantle tissues from *C. gibbosum* (*n* = 4) and whole *T. hamnerorum* individuals (*n* = 2) were fixed for 2 h at 4 °C in a buffered seawater solution of 2% paraformaldehyde, 2.5% glutaraldehyde (Karnovsky's Fixative) obtained from Electron Microscopy Sciences (Hatfield, PA, USA). Tissue samples were then transferred to PBS and kept at 4 °C till embedding. Paraffin-embedded samples were sectioned at 5 µm and mounted on Superfrost Plus (Fisher Scientific, St. Louis, MO, USA) microscope slides, deparaffined, and then hydrated with normal mouse serum in 10 mM phosphate buffered saline (pH 7.5). Sections were incubated for 1 h at room temperature with the mAb C219 diluted (2 µg/mL) in Primary Antibody Diluent (Signet). Antibody binding was visualized with a commercial avidin-biotin-peroxidase kit using diaminobenzidine tetrahydrochloride (DAB) as the chromogen (Vectastain® Elite ABC Kit, Vector Laboratories, Inc., Burlingame, CA, USA) according to the manufacturer's instructions. Specific staining was evaluated by light microscopy by comparison of mAb C219 stained and stained serial sections with normal mouse serum.

2.8. In vivo dye transport assays

MK571 was purchased from Cayman Chemical (Ann Arbor, MI, USA). Calcein-acetoxymethylester (C-AM) was purchased from Axxora (San Diego, CA, USA). Calcein was purchased from Invitrogen (Carlsbad, CA, USA). Verapamil was purchased from MP Biomedicals (Solon, OH, USA). Concentrated stocks of MK571 and C-AM were prepared in DMSO and were sufficiently concentrated so that the DMSO volume did not exceed 0.5% of the experimental volume.

ABC transporter activity was measured by the accumulation of calcein in whole *T. hamnerorum* individuals. Calcein-AM (C-AM) is a non-fluorescent substrate for both MRP and P-gp. Any C-AM entering the cell, if not transported out immediately by either ABC transporter, is rapidly hydrolyzed by intracellular esterases to form calcein, a fluorescent membrane-impermeable molecule (Essodaigui et al., 1998). Therefore, exposure to inhibitors of ABC transporter activity results in high calcein accumulation and increased fluorescence, while reduced intracellular accumulation of calcein and fluorescence is observed when transporter activity is increased.

Nudibranchs maintained in the laboratory were removed from their host, *G. ventalina*, and allowed to depurate for 4 h in a beaker of sterile-filtered seawater with aeration. Five similarly sized *T. hamnerorum* (~7 mm in length) were placed in each well of a 24-well plate and incubated for 2 h in 3 mL of 500 nM C-AM resuspended in sterile-filtered seawater. Incubations were performed in the presence or absence of two inhibitors at two concentrations. MK571 specifically inhibits MRP transport activity, while verapamil inhibits P-gp transport activity. Nudibranchs were then rapidly washed two times with sterile-filtered seawater and homogenized for 30 s using an electric drill fitted with a Teflon pestle in a 2 mL Wheaton glass homogenizer containing 125 µL of ethanol. The homogenate (~175 µL) was transferred to a 1.5 mL tube and centrifuged at room temperature for 7 min at 6.1 relative centrifugal force (RCF). Eighty-five microliters of supernatant was transferred to a 96-well black plate containing a clear bottom to minimize light scattering. The level of calcein in the resulting supernatant was measured fluorimetrically ($\lambda_{\text{ex}} = 485 \text{ nm}$, $\lambda_{\text{em}} = 530 \text{ nm}$) using a Cytofluor 2300 plate reader (Millipore). The ratio of intracellular calcein fluorescence in the absence and presence of MK571 or verapamil was used as a measure of the activity of *T. hamnerorum* MRP and P-gp transporters. Statistical analysis was performed using a two sample *t* test of the means with a significance level of 5%. *In vivo* dye assays were unable to be performed with *C. gibbosum* tissues due to the highly variable natural auto-fluorescence of the digestive gland in the absence of C-AM.

3. Results

3.1. Identification and phylogenetic analysis of ABC transporters

To define the range of ABC transporter genes expressed in molluscan tissues, a RT-PCR cloning approach with pooled mRNA samples and degenerate oligonucleotide primers was used initially to identify P-gp and MRP fragments with sequence corresponding to the conserved nucleotide binding domain of ABC transporter genes. BLASTX (NCBI) comparison of translated cDNAs indicated homology to mammalian ABCB and ABCC proteins. Initial gene fragment sizes were as follows: *C. gibbosum* – CgMRP isoform 1 and CgMRP isoform 2, 168 bp; *C. gibbosum* – CgPgp isoform 1 and CgPgp isoform 2, 216 bp; *T. hamnerorum* – ThMRP isoform 1 and ThMRP isoform 2, 170 bp; *T. hamnerorum* – ThPgp isoform 1 and ThPgp isoform 2, 71 and 182 bp respectively. Specific oligonucleotide primers designed to these initial fragments were used in 5' and 3' RACE reactions to obtain the eight partial MXR sequences ranging in length from 390 to 4647 bp (Table 1).

Deduced amino acid alignments of the gastropod MRPs and P-gps generated from nucleotide consensus sequences are shown in Figs. S1

Table 1
Summary of MXR cDNAs from *Cyphoma gibbosum* and *Tritonia hamnerorum*.

Gene subfamily	Species	Tissue	Gene designation	Length of cloned fragment		GenBank accession no.
				Nucleotide (bp)	Amino acid (a.a.)	
ABCB	<i>Cyphoma gibbosum</i>	Digestive gland	P-gp isoform 1	1755	584	EU487190
			P-gp isoform 2	1029	342	EU487191
	<i>Tritonia hamnerorum</i>	Whole animal	P-gp isoform 1	390	130	EU487196
			P-gp isoform 2	410	136	EU487197
ABCC	<i>Cyphoma gibbosum</i>	Digestive gland	MRP isoform 1	2853	951	EU487192
			MRP isoform 2	554	184	EU487193
	<i>Tritonia hamnerorum</i>	Whole animal	MRP isoform 1	4647	1549	EU487194
			MRP isoform 2	2297	765	EU487195

and S2 in the Supplementary data. ThMRP isoform 1 amino acid alignments with human ABCC sequences revealed a nearly full-length coding sequence of 1549 amino acids. A partial sequence encoding 951 amino acids, including the putative start codon and first two transmembrane spanning domain/nucleotide binding domain, was obtained for CgMRP isoform 1. Polypeptide lengths of 184 and 765 amino acids were obtained for CgMRP isoform 2 and ThMRP isoform 2, respectively, both of which aligned to the first nucleotide binding domain (Fig. S1, Supplementary data). An alignment of gastropod P-gp amino acid sequences with human ABCB1 revealed that both *Tritonia* P-gp sequences align with the first nucleotide binding domain, while *Cyphoma* P-gp sequences cover the second half of the protein, including the stop codon (Fig. S2, Supplementary data). In addition, hydrophobicity plots (Kyte and Doolittle, 1982) of *C. gibbosum* and *T. hamnerorum* ABC transporters agreed well with human ABCB1 and ABCC3 models (Figs. S3, S4 in the Supplementary data), increasing our confidence in the assignment of molluscan transmembrane and nucleotide binding domains.

Phylogenetic analysis of the deduced amino acid sequences revealed that the gastropod ABC transporters belong to the ABCB and ABCC subfamilies (Fig. 1). We constructed a phylogeny of ABCB and ABCC subfamilies in order to better define the relationships among proteins from humans, gastropods and a variety of other invertebrates (Fig. 2). There is strong Bayesian and ML support (1.0 and 99%, respectively) for the monophyly of these two subfamilies, and sequences within subfamilies are more similar to each other (Mean \pm S.D. pairwise similarity of ABCB subfamily = $48 \pm 8\%$; ABCC subfamily = $54 \pm 7\%$) than sequences of the other subfamily (Mean \pm S.D. pairwise similarity = $29 \pm 3\%$).

Within the ABCB subfamily, *Tritonia* P-gp isoform 1 and *Cyphoma* P-gp isoform 2 are embedded within a well-supported clade (0.99 Bayesian posterior probability) that includes sequences from human (ABCB2, ABCB3, ABCB8, ABCB9 and ABCB10) and a *Mytilus californianus* ABCB-like protein. *Tritonia* P-gp isoform 2 and *Cyphoma* P-gp isoform 1 cluster with each other (0.91 Bayesian posterior probability) and sequences from human (ABCB1, ABCB4, ABCB5, and ABCB11) and the bivalve *Brachidontes pharaonis* (1.0 Bayesian posterior probability).

Within the ABCC subfamily, two *Tritonia* (ThMRP isoform 1 and ThMRP isoform 2) and *Cyphoma* (CgMRP isoform 1) sequences cluster significantly with human (ABCC1, ABCC2, ABCC3 and ABCC6), *Drosophila* (CG6214) and *Mytilus californianus* sequences (1.0 and 48% by Bayesian and ML analysis, respectively). *Cyphoma* isoform 2 is embedded within a clade with human sequences (ABCC5, ABCC11, and ABCC12).

3.2. Quantitative RT-PCR analysis of *Cyphoma* ABCC (MRP-1) expression

Quantitative RT-PCR analysis was used to investigate the constitutive and inducible expression of MRP isoform 1 transcripts in *Cyphoma* after dietary exposure to several gorgonian species with varying allelochemical profiles. *Cyphoma* MRP isoform 1 was selected for expression analysis because it is found within a well-supported clade that contains both human MRP1 and MRP2 (ABCC1 and ABCC2, respectively), known for their ability to transport a range of

xenobiotics and phase II metabolites, including glutathione-conjugated substrates (Keppler et al., 1997; Nies and Keppler, 2007; Zimmermann et al., 2008). This is particularly relevant to the present study because *Cyphoma* digestive gland tissues constitutively express high levels of the glutathione-conjugating enzyme, glutathione S-transferase, likely necessitated by the presence of numerous potent inhibitors and/or substrates in its gorgonian diets (Whalen et al., 2008; Whalen et al., 2010).

Digestive gland CgMRP isoform 1 transcripts were measured by quantitative RT-PCR from adult *Cyphoma gibbosum* allowed to feed ad libitum for 4 days on one of seven gorgonian diets or a control diet lacking gorgonian chemistry. MRP isoform 1 transcripts were constitutively expressed in *Cyphoma* digestive gland and the expression was not affected by gorgonian diet (Supplemental Table S3, Fig. 3). MRP isoform 1 transcript expression was also measured in time-zero individuals to determine if collection location had any influence on gene expression. MRP isoform 1 expression was found to significantly differ in time-zero snails as a function of reef location (ANOVA, $F_{4,26} = 2.74$, $p = 0.05$, Fig. 4 and Table S4 in the Supplementary data), but did not significantly differ among snails allowed to feed on a control diet for 4 days (ANOVA, $F_{4,28} = 0.637$, $p = 0.640$).

3.3. Expression and localization of ABCB1 (P-gp)

Immunochemical detection of P-gp-like enzymes in molluscan tissue homogenates with the monoclonal antibody C219 revealed the presence of immunoreactive bands only for *Tritonia* homogenates. The antibody recognized a distinct band of expected size, 170 kDa (Juliano and Ling, 1976), and two larger diffuse bands, >200 kDa, possibly representing differentially glycosylated isoforms of P-gp (Schinkel et al., 1993) or the presence of multiple proteins in *Tritonia* (Fig. 5). However, similar immunoreactivity was absent for *Cyphoma* digestive gland homogenates. Based on deduced amino acid sequences, the lack of reactivity in *Cyphoma* homogenates is somewhat surprising considering that at least one of the *Cyphoma* proteins (CgPgp isoform 1) contains the epitope VQEALD, among those recognized by C219. Although C219 recognizes this epitope containing Glu (E), it does so less strongly than the epitope containing Ala (A) (VQAALD) (Georges et al., 1990). The partial *Tritonia* P-gp isoform 1 sequence contains the Ala at this position (VQAALD), which may explain the difference in reactivity between molluscan species. Alternatively, or in addition, there may be differences in P-gp expression that contribute to the differential recognition by C219 in these immunoblot studies.

The cellular localization of P-glycoprotein was examined in fixed, paraffin-embedded molluscan tissue sections probed with mAb C219. P-glycoprotein expression was detected in the apical tips of the ciliated columnar epithelia cells lining the lumen of the midgut in *T. hamnerorum* (Fig. 6). In the non-distended midgut, immunohistochemical staining helps to accentuate the folded nature of this heavily ciliated epithelia (Millott, 1937; Morse, 1968). Faint staining was also noted in goblet-like cells found on the nudibranch's epidermis, which may have a role in mucus secretion (Fig. 6A). In contrast to the staining in *T. hamnerorum* tissues, immunoreactive protein was not

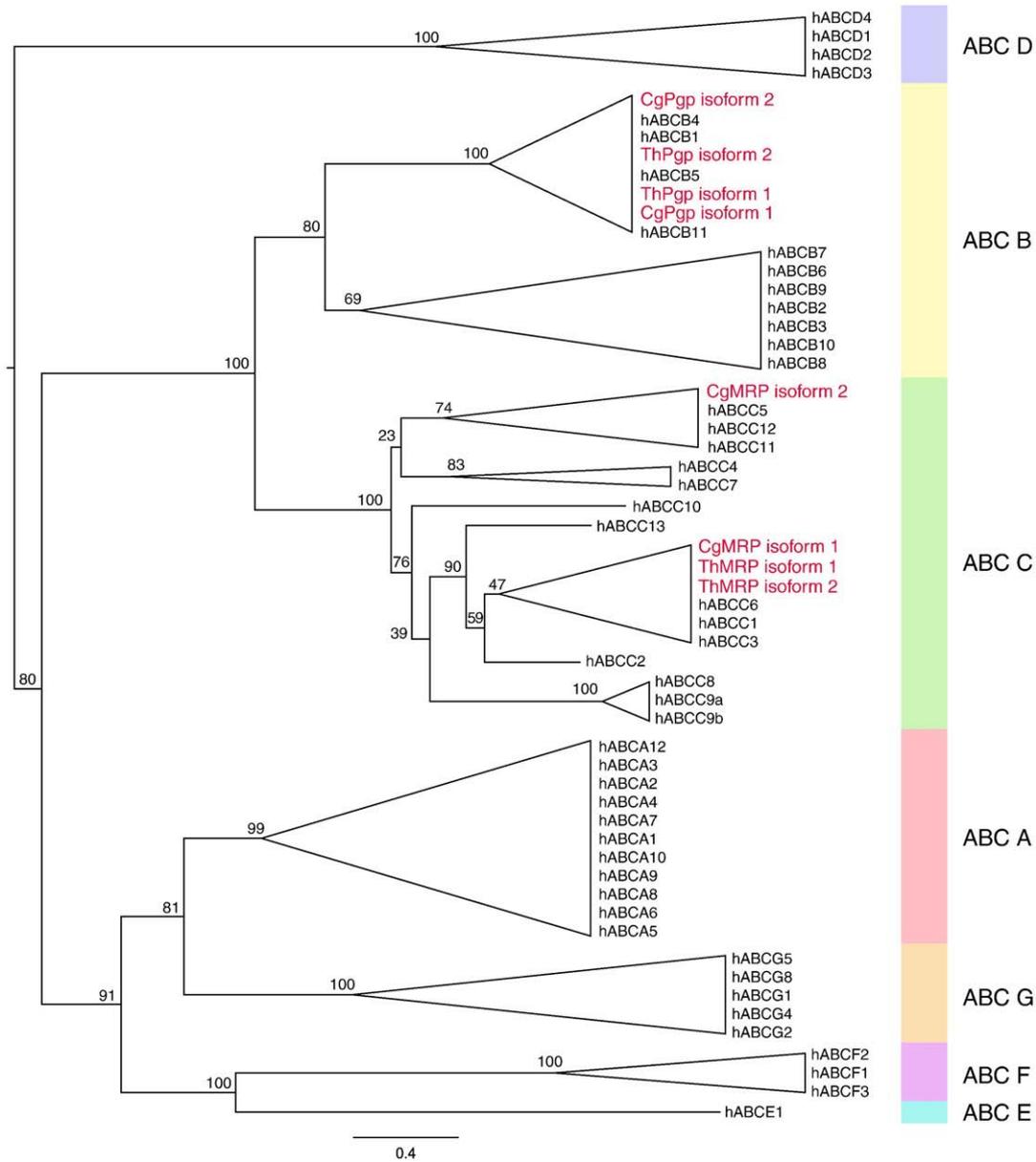


Fig. 1. Phylogenetic relationships of *Cyphoma*, *Tritonia* and human ABC transporters. Maximum likelihood (ML) trees were constructed as described in the Materials and methods. Out of a possible 3450 positions, 1821 positions (or 52.8%) were used to construct the final tree. Molluscan sequences are highlighted in red. Values at branch points represent ML bootstrap values calculated with 100 replications. Triangles represent portions of the tree that were collapsed due to poor resolution of the taxa within each clade as evidenced by bootstrap analysis. GenBank sequences in the tree include: Human ABCA proteins (ABCA1, NP_005493.2; ABCA2, NP_001597.2; NP_001597.2; ABCA3, NP001080.2; ABCA4, NP_000341.2; ABCA5, NP_061142.2; ABCA6, NP_525023.2; ABCA7, NP_061985.2; ABCA8, NP_009099.1; ABCA9, NP_525022.2; ABCA10, NP_525021.3; ABCA12, NP_775099.2), human ABCB proteins (ABCB1, NP_000918.2; ABCB2, NP_000584.2; ABCB3, NP_000535.3; ABCB4, NP_000434.1; ABCB5, NP_848654.3; ABCB6, NP_005680.1; ABCB7, NP_004290.2; ABCB8, NP_009119.2; ABCB9, NP_982269.1; ABCB10, NP_036221.1; ABCB11, NP_003733.2), human ABCC proteins (ABCC1, NP_004987.2; ABCC2, NP_000383.1; ABCC3, NP_003777.2; ABCC4, NP_005836.2; ABCC5, NP_005679.2; ABCC6, NP_001162.3; ABCC8, NP_000343.2; ABCC9 a/b, NP_005682.2, NP_064693.2; ABCC10, NP_258261.2; ABCC11, NP_115972.2; ABCC12, NP_150229.2; ABCC13, EAX10058.1), human ABCD (ABCD1, NP_000024.2; ABCD2, NP_005155.1; ABCD3, NP_002849.1, ABCD4, NP_005041.1), human ABCE (ABCE1, NP_002931.2), human ABCF (ABCF1, NP_001020262.1; ABCF2, NP_009120.1; ABCF3, NP_060828.2), human ABCG (ABCG1, NP_997057.1; ABCG2, NP_004818.2; ABCG4, NP_071452.2; ABCG5, NP_071881.1; ABCG8, NP_071882.1), *Cyphoma gibbosum* MRP isoform 1 (EU487192), MRP isoform 2 (EU487193), P-gp isoform 1 (EU487190), P-gp isoform 2 (EU487191), *Tritonia hamnerorum* MRP isoform 1 (EU487194), MRP isoform 2 (EU487195), P-gp isoform 1 (EU487196), P-gp isoform 2 (EU487197).

detected in *C. gibbosum* mantle or digestive gland tissues. Negative control sections of *T. hamnerorum* (Fig. 6E) or *C. gibbosum* (data not shown) tissues stained with normal mouse serum displayed no immunolabelling.

3.4. In vivo calcein efflux transport assay

In vivo examination of transporter activity in *Tritonia* was assessed using the calcein efflux assay. The calcein assay is a functional diagnostic

method developed to quantify MXR-mediated efflux activity in cells and tissues. Non-fluorescent calcein-AM rapidly traverses cell membranes where it can be converted to fluorescent calcein by non-specific esterases or extruded by P-gp and MRP before intracellular conversion to the non-MXR substrate. High levels of MXR transport activity can prevent the accumulation of free calcein in the cell, resulting in minimal fluorescent calcein accumulation. Transporter-specific MXR-mediated efflux can be measured by the use of specific MRP (MK571) and P-gp (verapamil) inhibitors. If transport activity is minimal, the transport

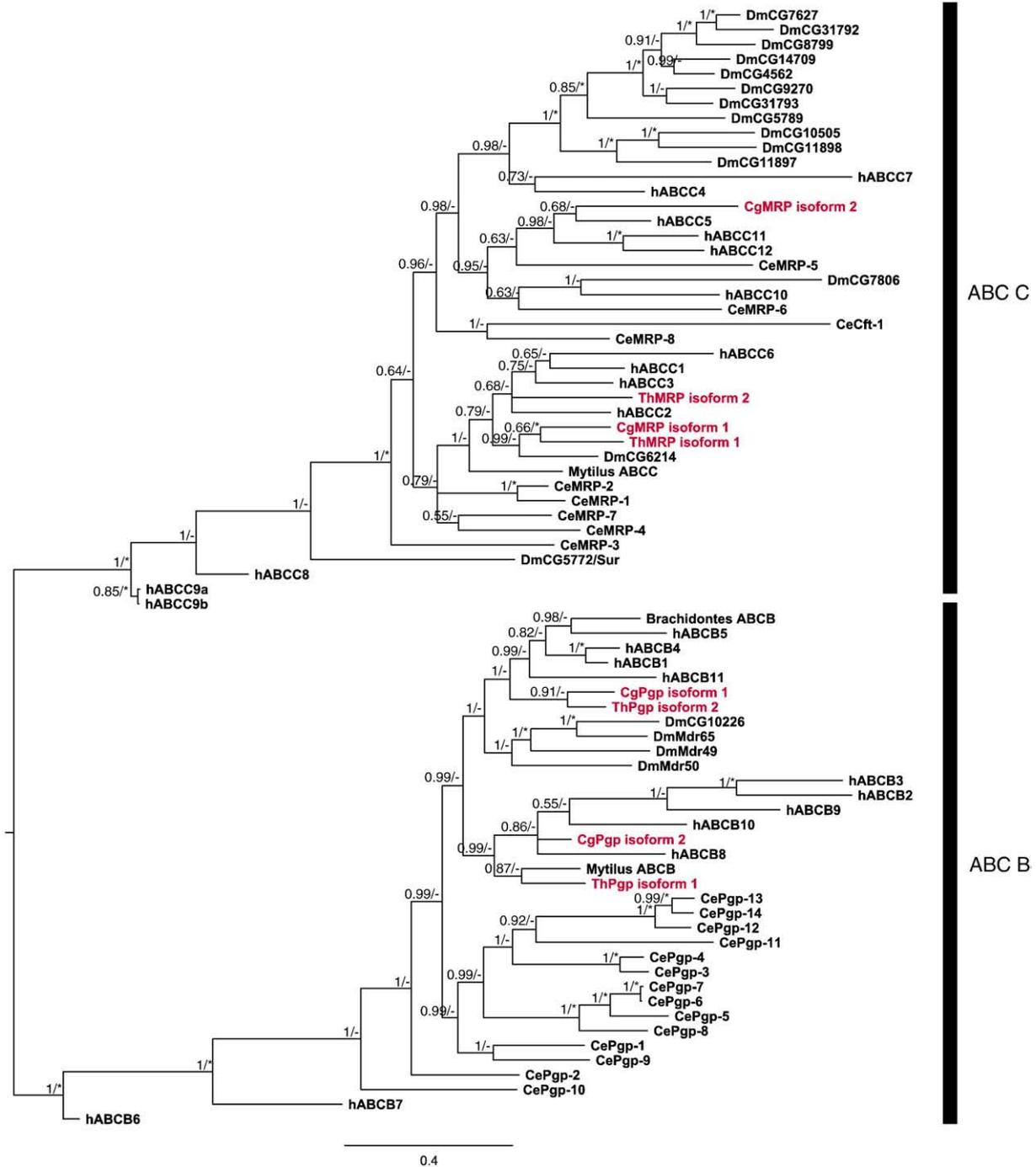


Fig. 2. Phylogenetic relationships of *Cyphoma* and *Tritonia* ABC proteins with human and invertebrate ABCB and ABCC subfamilies. The Bayesian phylogeny is presented with posterior probability and ML bootstrap support indicated for each branch (e.g., Bayesian/ML). ML bootstrap support greater or equal to 70% is indicated by an asterisk (*), less than 70% is indicated by a dash (-). Gastropod sequences are highlighted in red. GenBank sequences included in the tree can be found in Supplemental File S2.

inhibitor will have limited or no effect resulting in little change in cell fluorescence. However, if transport activity is significant, the presence of an inhibitor will increase calcein accumulation, as indicated by intracellular fluorescence, compared to incubations without the inhibitor present.

Changes in fluorescence intensity of calcein in *Tritonia* tissues incubated in the presence of 500 nM extracellular C-AM with and without varying concentrations of inhibitors are shown in Fig. 7. The addition of verapamil or MK571 to the seawater containing *Tritonia* significantly increased calcein accumulation compared to DMSO

controls. These results indicate that MXR-mediated efflux activity prevents calcein accumulation in *Tritonia* tissues and that inhibiting this activity causes calcein-AM to be retained and hydrolyzed to calcein. Calcein accumulation varied depending on the inhibitor used and its concentration. Increasing verapamil concentration to 25 μ M did not cause a measurable increase in calcein accumulation compared to 5 μ M of verapamil, suggesting that the lower concentration of verapamil is sufficient to block the majority of P-gp transporters. In contrast, 30 μ M MK571 resulted in a further 57% increase in calcein accumulation compared to that caused by 5 μ M

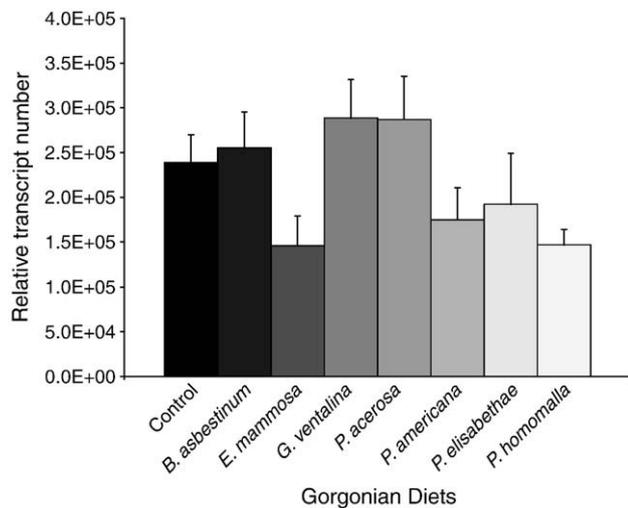


Fig. 3. Mean MRP isoform 1 transcript expression among *C. gibbosum* individuals feeding on a gorgonian or control diet for 4 days. Bars represent the mean transcript expression (\pm SE) of snails feeding on *B. asbestinum* ($n=13$), *E. mammosa* ($n=12$), *G. ventalina* ($n=13$), *P. acerosa* ($n=10$), *P. americana* ($n=12$), *P. elisabethae* ($n=6$), *P. homomalla* ($n=11$) or a control diet ($n=33$). The relative number of transcripts per 0.2 μ g of poly(A)+ RNA was calculated from the standard curve and normalized by a β -actin correction factor. Results of a two-way ANOVA indicate no differences in MRP isoform 1 expression in snails feeding on different diets.

MK571, and the calcein accumulation was greater than the maximal level obtained in the presence of verapamil. This difference in the absolute level of calcein accumulation suggests that MRP transporters are either more highly expressed or more efficient at exporting C-AM in *Tritonia* tissues.

4. Discussion

Our knowledge of the function of ABC transporters in marine invertebrates is mostly limited to the response of these proteins under anthropogenic stress conditions (Kurelec, 1992; Minier et al., 1993; Smital et al., 2000; Smital et al., 2003; Kingtong et al., 2007). However, for marine consumers that regularly feed on prey/hosts laden with natural toxins, efflux transporters may play a critical role in regulating the absorption of these allelochemicals, thereby influencing diet selection. This study is the first to examine the expression and activity of ABC transporters from two predatory molluscs that feed solely on chemically defended prey.

4.1. Molluscan P-glycoproteins

Using a combination of molecular approaches, four partial cDNA sequences of P-gps related to multidrug transporters in vertebrates were identified from the digestive gland of *Cyphoma gibbosum* (CgPgp isoform 1, CgPgp isoform 2) and whole *Tritonia hamnerorum* (ThPgp isoform 1, ThPgp isoform 2). ThPgp isoform 2 and CgPgp isoform 1 clusters with human ABCB1, ABCB4, ABCB5 and ABCB11 genes. ABCB1 (MDR1 or P-gp1) is possibly the most well studied of all the multidrug transporters and is known for its promiscuous transport of hydrophobic substrates, including xenobiotics, lipids, sterols, and chemotherapeutic agents derived from natural products (Choudhuri and Klaassen, 2006). Human ABCB4 (MDR3) and ABCB11 are both expressed in the liver and are involved in the secretion of bile acids (Gerloff et al., 1998; Oude Elferink and Paulusma, 2007), while ABCB5 is hypothesized to function as a drug resistance mediator, similar to ABCB1, and is primarily expressed in the epidermis (Frank et al., 2005). Acquisition of complete ABCB sequences would help to better define the relationships of the molluscan P-gps with particular ABCB members. Nevertheless, the phylogenetic analysis presented here clearly groups these molluscan transporters with those ABCB members known to mediate xenobiotic resistance.

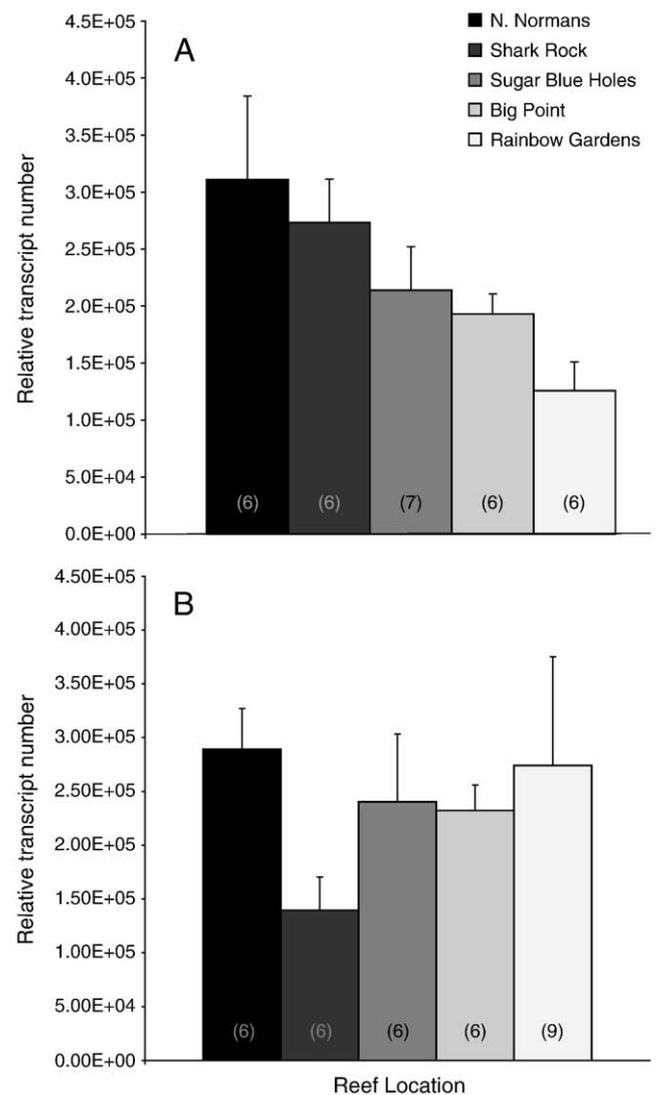


Fig. 4. Mean MRP isoform 1 transcript expression among time-zero and 4 day control diet fed *C. gibbosum* collected from five reefs. (A) Time-zero snails; mean MRP isoform 1 expression (\pm SE) in snails ($n=31$ snails) collected from five reefs and immediately dissected to preserve reef-specific gene expression signals. (B) Control snails; mean MRP isoform 1 expression (\pm SE) in snails ($n=33$ snails) collected from five reefs and fed a control diet (e.g., alginate + squid powder) for 4 days. Values in parentheses indicate the number of replicate snails examined per reef. The relative number of transcripts per 0.2 μ g of poly(A)+ RNA was calculated from the standard curve and normalized by a β -actin correction factor.

Tritonia P-gp isoform 1 and *Cyphoma* P-gp isoform 2 appear to cluster with human half transporters ABCB2(TAP1), ABCB3(TAP2), ABCB8(M-ABCB1), ABCB9 and ABCB10(M-ABCB2) genes. Human ABCB2 and ABCB3 encode half transporters that form a heterodimer and transport peptides derived from proteasomal degradation from the cytosol into the endoplasmic reticulum for loading onto major histocompatibility complex (MHC) class I molecules (Sturm et al., 2009). Human ABCB9 is homodimeric and shares structural similarities to ABC2/3, however its function has yet to be defined, while human mitochondrial transporters ABCB8 and ABCB10 function in iron metabolism and transport of Fe/S protein precursors. It is therefore possible that these gastropod isoforms are also half transporters; however, since invertebrates lack the mammalian adaptive immune response, the functional role of these molluscan transporters is not so obvious.

Western blot analysis of molluscan tissues with the mAb C219, which recognizes both human MDR1 (ABCB1) and MDR3 (ABCB4) proteins (Van den Elsen et al., 1999), suggested that homologs of human MDR

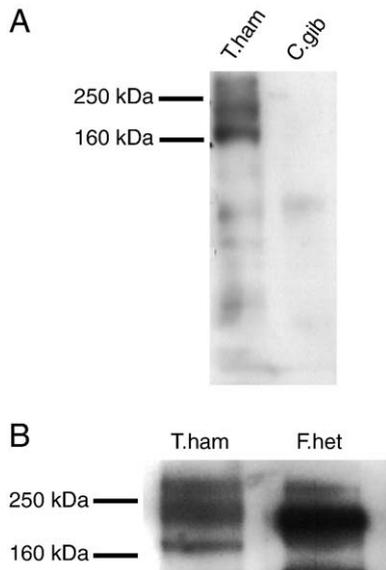


Fig. 5. Detection of P-glycoprotein proteins in molluscan tissue homogenates. (A) Western blot was probed with anti-P-glycoprotein mAb C219 which reacted with one sharp band at 170 kDa and two diffuse bands at >170 kDa in *T. hamnerorum* whole cell lysates. No bands were detected in *C. gibbosum* digestive gland lysates. (B) *Fundulus heteroclitus* liver cell lysates were used as a positive control.

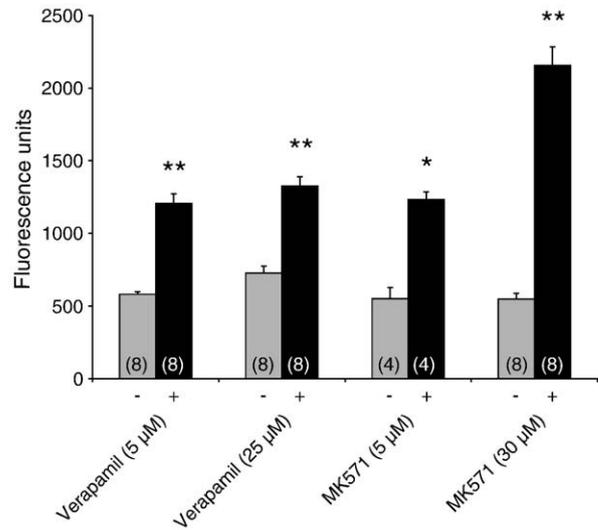


Fig. 7. Change in intracellular fluorescence in *T. hamnerorum* incubated with MRP (MK571) and P-gp (verapamil) inhibitors compared to untreated controls. Bars represent mean fluorescence (\pm SE) of *T. hamnerorum* tissue homogenates from four or eight replicate wells. Number of replicate wells for DMSO control (grey bars) and inhibitor exposed (black bars) are indicated in the parentheses. All inhibitor exposures resulted in a significant increase in calcein accumulation. MK571 at 30 μ M exposure resulted in the greatest inhibition of transport activity in *T. hamnerorum*. * $P=0.001$, ** $P<0.001$.

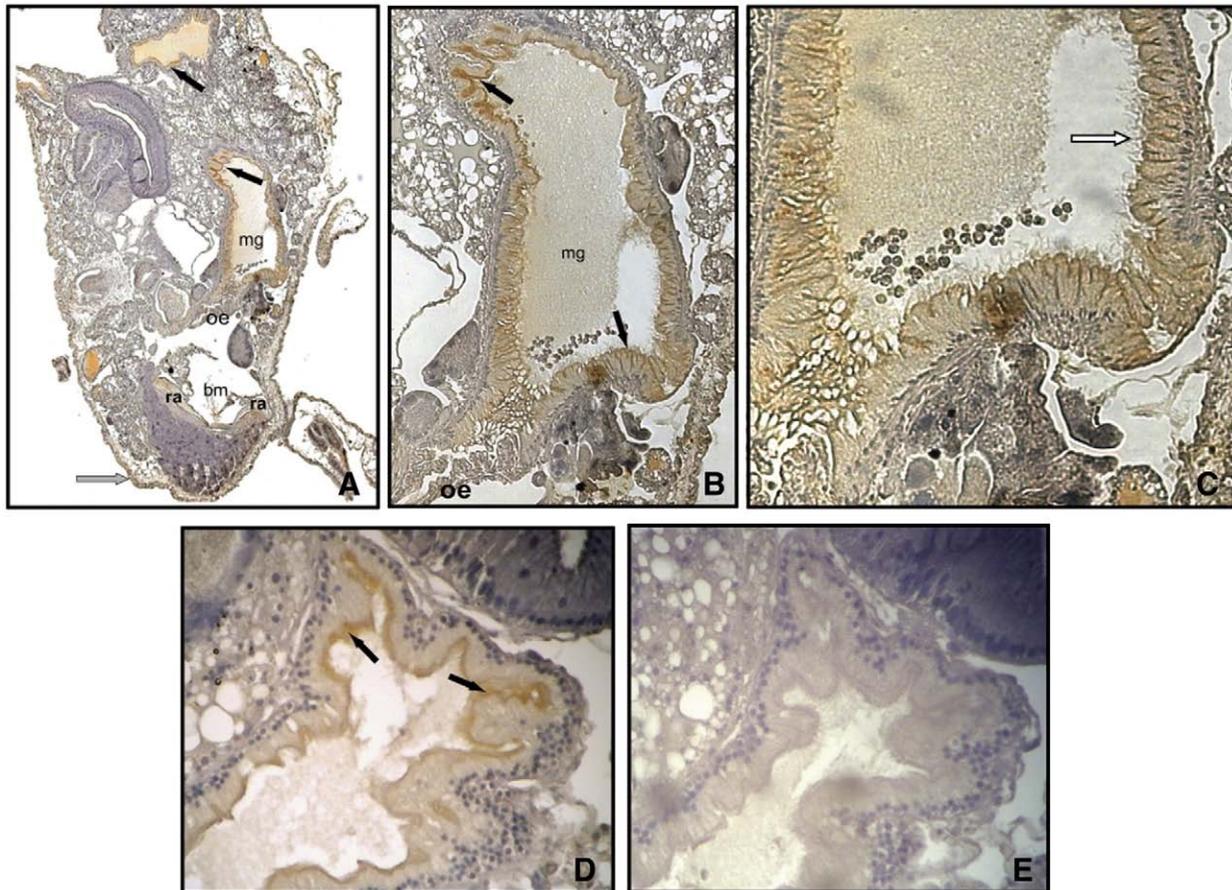


Fig. 6. Immunohistochemical analysis of *T. hamnerorum* tissues. Tangential sections through *T. hamnerorum* probed with mAb C219. Orange-brown staining indicates immunoreactive protein. (A) Black arrows indicate intense staining by C219; grey arrow indicates lighter staining by C219 along the epidermis; *bm*, buccal mass; *mg*, midgut; *oe*, esophagus; *ra*, radula; (50 \times). (B) Magnification (100 \times) of esophagus and midgut. (C) Further magnification (200 \times) of the midgut ciliated columnar epithelia; cilia indicated by white arrow. (D, E) C219 probed tissue section (D) and unlabeled serial section (E) depicting P-glycoprotein localization to the apical tips of the midgut epithelium (indicated by black arrows) (200 \times).

proteins are expressed in *Tritonia* tissues but not in *Cyphoma* digestive gland. The apparent disparity in P-gp expression between the two predatory molluscs may reflect differences in their foraging behavior. A previous study showed that specialist herbivores that consumed a diet rich in allelochemicals had a higher intestinal P-gp capacity in comparison to sympatric generalists that were unable to tolerate the toxin-laden diet (Green et al., 2004). These results suggest that dietary toxins could be handled differently depending on consumer experience.

Deduced amino acid sequences from two partial *Cyphoma* P-gps indicate that at least one of the forms (CgPgp isoform 1) contains the correct epitope (VQEALD) in its nucleotide binding domain and therefore would be recognized by C219 if expressed in the digestive gland. It is possible that CgPgp isoform 1 is expressed in tissues other than the digestive gland; however, immunohistochemical analysis of whole *Cyphoma* tissue sections with C219 did not detect any immunoreactive protein. Based on sequence data, multiple forms of P-gp are expressed in *Cyphoma* digestive gland, yet their detection may be limited by the reactivity of the antibody used here, suggesting that western blots should be interpreted with caution. In contrast, *Tritonia* tissue homogenates displayed a distinct band at 170 kDa, in agreement with human MDR proteins (Choudhuri and Klaassen, 2006) and two diffuse bands at >200 kDa. Similar multiple banding patterns (i.e., 170 kDa, and >200 kDa banding) have also been observed in the gill tissue of mussels *Mytilus californianus*, *M. galloprovincialis*, and *M. edulis* and oysters *Crassostrea gigas* and *C. virginica* (i.e., >200 kDa) (reviewed in Bard 2000). Protein bands >200 kDa may represent post-translational modifications or differential glycosylation states, not uncommon among mammalian P-glycoproteins (Schinkel et al., 1993).

Immunohistochemical staining of *Tritonia* tissues by C219 indicated that P-gp proteins were localized to the apical epithelial of the midgut and to a lesser extent in the epidermis. The location of P-gp in *Tritonia* digestive tissues is in agreement with the localization of mammalian ABCB1 on the apical (or luminal) surface of polarized epithelia of many tissues, such as the gastrointestinal tract, kidney proximal tubules and biliary hepatocytes (Klein et al., 1999). Based on the location of mammalian ABCB1 in barrier tissues coupled with its function as a unidirectional transporter of a range of toxic substrates, it is likely that the physiological role of ABCB1 and its orthologs is to protect cells and ultimately organisms against toxic compounds (Schinkel et al., 1994; Schinkel et al., 1997). The two tissue types observed to exhibit the greatest P-gp expression in *Tritonia*, the gut epithelia and the epidermis, correspond to those tissues that would be exposed to the greatest concentration of gorgonian allelochemicals, due to the fact that *Tritonia* both resides on and consumes its gorgonian host.

Interestingly, this nudibranch is able to selectively sequester a furano-germacrene feeding deterrent from its gorgonian host, *Gorgonia ventalina*, and concentrate this chemical defense four-fold (dry mass basis) relative to the gorgonian (Cronin et al., 1995). While the exact mechanism of sequestration in nudibranch tissues is unknown, recent studies have demonstrated that mammalian MXR transporters can reduce the intracellular concentration of toxins by actively sequestering them in subcellular compartments, away from vulnerable cellular targets (Van Luyn et al., 1998; Molinari et al., 2002; Rajagopal and Simon, 2003; Ifergan et al., 2005) or within certain organs (Jonker et al., 2005). Furthermore, MXR members can have different activity profiles depending on membrane location (i.e., plasma membrane versus lysosomal membrane), which may be linked to the lipid environment or post-translational modification of the transporter that can occur within subcellular compartments (Rajagopal and Simon, 2003). This intracellular sequestration phenomenon has also been observed in mussel blood cells, whereby the intracellular accumulation of the fluorescent P-gp substrate Rhodamine B in lysosomes could be reversed if incubated with verapamil (Svensson et al., 2003). In addition, MXR-mediated

subcellular localization is suspected to be responsible for the sequestration of the marine toxin, okadaic acid in the mussel *Mytilus edulis* (Svensson et al., 2003). Transporters have also been suggested as a mechanism by which herbivorous insects can sequester unmetabolized host plant toxins in diverticular pouches, defensive glands or hemolymph (Sorensen and Dearing, 2006). Chemical analysis of nudibranch tissues indicated that diet-derived allelochemicals are selectively accumulated in the mantle border and in mucus secretions (Pawlik et al., 1988; Garcia-Gomez et al., 1990; Fontana et al., 1994; Avila and Paul, 1997). In *Tritonia*, the precise tissue location of toxin sequestration is unknown; however, P-gp immunoreactive protein was detected on this nudibranch's epidermis, a location consistent with the site of toxin accumulation in other nudibranch species. Further work is needed to determine whether ABCB1-like proteins within the dermis of nudibranchs participate in toxin accumulation; nevertheless, the presence of ABC transporters in these specialist consumers may not only function as a protective mechanism against cytotoxicity, but may have an added advantage of providing these consumers with their own chemical protection.

The *in vivo* activity of MXR transporters was investigated in *Tritonia* using ABC subfamily-specific transport inhibitors capable of blocking the P-gp- and MRP-mediated efflux of C-AM. Both inhibitors resulted in increased dye accumulation in *Tritonia* tissues, which is consistent with previous competitive dye transport assays reporting MXR-mediated transport activity in a variety of marine invertebrates (grass shrimp (Finley et al., 1998), worm (Toomey and Epel, 1993), sponge (Muller et al., 1996), oyster (Keppler, 1997), mussel (McFadzen et al., 2000), sea urchin (Hamdoun et al., 2004)). Varying inhibitor concentration did not increase P-gp-mediated calcein accumulation, but did cause a substantial impact on MRP-mediated calcein accumulation. The greater level of calcein accumulation in *Tritonia* exposed to MRP inhibitor suggests that MRP-mediated efflux may contribute more to xenobiotic resistance than P-gp in *Tritonia*.

4.2. Molluscan multixenobiotic resistance-associated proteins

While both subfamilies of transporters confer multixenobiotic resistance, the substrate selectivities of P-gp and MRP differ markedly. P-gp transports neutral or mildly positive lipophilic compounds, while MRP substrates are lipophilic anions capable of transporting a range of substrates including glutathione (GSH), glucuronide, or sulfate conjugates of phase II detoxification reactions (Kruh and Belinsky, 2003). Phylogenetic analysis of molluscan MRP sequences obtained here indicate that CgMRP isoform 1, ThMRP isoform 1 and ThMRP isoform 2 fall within a well-supported clade containing the mammalian ABCC1, ABCC2, ABCC3 and ABCC6 proteins, known for their ability to transport a variety of glutathione conjugates including natural product cancer drugs and prostaglandins (Evers et al., 1997; de Waart et al., 2006). The Caribbean gorgonian *Plexaura homomalla* is regularly consumed by *Cyphoma* despite the high concentration of prostaglandin A₂ esters (Gerhart, 1986), which function as potent feeding deterrents in this coral (Gerhart, 1984; Pawlik and Fenical, 1989). Recent evidence suggests that *Cyphoma* may be able to tolerate dietary prostaglandins and other lipophilic gorgonian compounds by conjugating them with GSH, catalyzed by glutathione S-transferases (GSTs). GSTs are highly expressed in the digestive gland of this predator, and a screening of gorgonian lipophilic extracts suggests that all gorgonian diets may contain substrates for *Cyphoma* GSTs (Whalen et al., 2010). Therefore, the constitutive expression of MRP isoform 1 in the digestive gland of *Cyphoma* may facilitate the efficient biliary excretion of putative glutathione conjugates of prostaglandins and other lipophilic compounds (Evers et al., 1997; Paumi et al., 2003; de Waart et al., 2006). MRP isoform 1 expression was also detected in the snails feeding on control diets lacking gorgonian allelochemicals, suggesting that these transporters may also be responsible for the export of physiological substrates, in addition to their roles in

multixenobiotic resistance. In contrast, MRP isoform 1 expression significantly varied in time-zero snails as a function of reef location. While it is difficult to conclusively pinpoint the cause of this reef-specific variation, the length of snail foraging time on a single colony or the presence of additional gorgonian species not tested in our feeding assays may have had an effect on MRP isoform 1 expression.

Phylogenetic analysis revealed that *Cyphoma* MRP isoform 2 is significantly divergent from the other molluscan MRPs identified in this study and falls into the clade containing human ABCC5, ABCC11 and ABCC12. All three of these transporters are at the early stages of investigation and their physiological functions are not well understood. However, studies indicate that they mediate the transport of antiretroviral nucleosides and lipophilic glutathione conjugates (Wijnholds et al., 2000; Kruh et al., 2007).

5. Conclusions

Mounting evidence suggests that ABC transporters likely regulate the absorption and subsequent distribution of natural toxins in marine organisms (Toomey and Epel, 1993; Toomey et al., 1996; Keppler and Ringwood, 2001; Eufemia et al., 2002). The present work describes the first efforts to identify MXR genes that may protect marine invertebrates that have adapted to exclusively feed on allelochemically-rich prey. Messenger RNA encoding MXR transporters is expressed in both generalist and specialist molluscan species and these transporters share sequence and organizational structure similar to MXRs of distantly related organisms, indicating the likelihood of shared function as natural product transporters between mammalian and molluscan orthologs. A second piece of evidence supporting the role of MXR proteins as dietary allelochemical efflux pumps comes from the results of immunohistochemical analyses in *Tritonia* tissues, where P-gp expression was highest in the tissues most vulnerable to allelochemical exposure. Additionally, *in vivo* studies of MXR activity using ABC subfamily-specific inhibitors provided added support for the presence and function of MXR transporters in *Tritonia*. However, for organisms where *in vivo* dye assays may not be feasible, real-time qPCR provided a highly robust and sensitive method for quantifying MXR isoform-specific expression. Constitutive expression of selected promiscuous MXR transporters may be advantageous to a generalist predator like *Cyphoma* that maintains a chemically diverse diet. Support for this hypothesis may come from screening gorgonian extracts in competitive substrate inhibition assays with transfected cell lines or isolated membrane vesicles overexpressing molluscan MXRs of interest. This initial screening approach would facilitate comparisons of substrate breadth between MXRs from generalist and specialist consumers and allow one to begin to test predictions about whether generalist MXRs possess greater structural and functional flexibility in their substrate specificity compared to specialists (Li et al., 2004).

The elucidation of the molecular mechanisms underlying consumer tolerance is a prerequisite to understanding the foraging behavior of marine consumers. This study provides a much needed first step in identifying putative environmental chemical stress genes capable of ameliorating allelochemical-induced toxicity. It is likely that both MRP and P-gp play significant roles in natural toxicant efflux in marine consumers. Future work aimed at elucidating those marine natural products capable of interacting with substrate-binding sites on MXRs will provide valuable insight into the evolution of ABC transporter-mediated consumer counter-defense mechanisms.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cbpc.2010.05.003.

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