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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.

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 mechanisms 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 overexpression 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 web 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; $n \sim 40$ 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