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Schistosoma mansoni express higher levels of multidrug resistance-associated protein 1 (SmMRP1) in juvenile worms and in response to praziquantel

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ABSTRACT

The ATP-binding cassette (ABC) superfamily of proteins comprises several ATP-dependent efflux pumps involved in transport of toxins and xenobiotics from cells. These transporters are essential components of normal physiology, and a subset is associated with development of multidrug resistance. P-glycoprotein (Pgp) and the multidrug resistance-associated proteins (MRPs) represent two classes of these multidrug resistance (MDR) transporters. MRP1 is one type of mammalian MRP, which preferentially transports anionic compounds and compounds detoxified by cellular enzymes such as glutathione-S-transferase. It also transports signaling molecules, including immunomodulators. In schistosomes, both Pgp and MRP substrates localize to the excretory system, a potentially attractive target for new antischistosomals. We have previously shown that expression of schistosome Pgp (SMDR2) is altered in worms exposed to praziquantel (PZQ), the current drug of choice against schistosomiasis, and is expressed at higher levels in worms from isolates with reduced PZQ susceptibility. We have also shown that PZQ interacts directly with SMDR2. Here, we examine the relationship between PZQ and SmMRP1, a *Schistosoma mansoni* homolog of mammalian MRP1. SmMRP1 RNA is differentially expressed in adult males and females, and levels increase transiently following exposure of adult worms to sub-lethal concentrations of PZQ. A corresponding, though delayed, increase in anti-MRP1-immunoreactive protein also occurs following exposure to PZQ. PZQ-insensitive juvenile worms express higher levels of both SmMRP1 and SMDR2 RNA than mature adults, consistent with the hypothesis that increases in levels of schistosome multidrug transporters may be involved in development or maintenance of reduced susceptibility to PZQ.

1. Introduction

Trematode flatworms of the genus *Schistosoma* are the causative agents of schistosomiasis, which affects approximately 200 million people worldwide, an estimated 90% of whom live in Africa [1]. As many as 280,000 deaths per year in Africa alone have been attributed to schistosomiasis [2], and schistosomiasis has been estimated to have a global disease impact of up to 70 million disability-adjusted life years (DALYs) lost annually [3], higher than estimates for malaria and comparable to those for HIV/AIDS [4]. Adult schistosomes living in the blood vessels of the host must be able to take up nutrients, but they also require mechanisms to eliminate their own toxic metabolites as well as compounds derived from the host [5]. These mechanisms likely include the use of multidrug transporters, cellular efflux pumps with broad substrate specificities.

The phenomenon of multidrug resistance (MDR) was initially found in mammalian tumor cells that had been selected for resistance to a single drug, but which also showed unexpected cross-resistance against several structurally unrelated compounds. The phenomenon is linked to increased drug efflux via particular members of the ATP-binding cassette (ABC) superfamily of transporters, including P-glycoprotein (Pgp), multidrug resistance-associated proteins (MRPs), including MRP1, breast cancer resistance protein (BCRP), and others [6,7]. The role of these transporters in normal cellular physiology is to remove or exclude xenobiotics and metabolic toxins, and they also play essential roles in a wide variety of physiological processes [8–10], including regulation of immune responses [11]. Several labs have found that multidrug transporter expression levels and allele frequencies are altered in anthelmintic-resistant populations of helminths, including schistosomes [12–20], and the potential role of these transporters in helminth and other parasite drug resistance has recently been reviewed [21–25].

The current drug of choice against schistosomiasis is praziquantel (PZQ), which is active against all schistosome species, shows minimal side effects, and is also effective against other trematode and cestode infections [26,27]. The value of PZQ has been demonstrated repeatedly in large-scale schistosomiasis control efforts in

Abbreviations: MDR, multidrug resistance; MRP, multidrug resistance-associated protein; PZQ, praziquantel; Pgp, P-glycoprotein; ABC, ATP-binding cassette.

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a variety of countries [28,29]. However, schistosomes show stage- and sex-dependent differences in susceptibility to PZQ [30–32]. Furthermore, with the mode of PZQ action remaining incompletely defined [33–35], the prospect of emerging resistance to PZQ is of particular concern [36,37].

Previously, we showed that *Schistosoma mansoni* adults transiently upregulate expression of SMDR2, a schistosome homolog of Pgp [38], in response to exposure to low concentrations of PZQ [20]. Additionally, worms from an Egyptian isolate with reduced PZQ sensitivity express dramatically higher levels of SMDR2 than do PZQ-susceptible worms [20]. We have also shown that PZQ is an inhibitor of rhodamine transport by SMDR2 expressed in mammalian cells, with an IC_{50} of 17.4 μM, and that BODIPY-PZQ is a substrate of SMDR2 [39]. These results, along with those from others showing PZQ-induced changes in localization of a fluorescent Pgp substrate [40,41], indicate that at least one MDR transporter responds to PZQ, and has expression levels correlated with reduced susceptibility to PZQ.

Mammalian MRP1 (ABCC1) is one of a set of nine human MRP genes [6,7], and shows some overlap in its spectrum of substrates with Pgp. However, unlike Pgp, it has preference for anionic compounds and for structurally diverse drugs and xenobiotics conjugated to glutathione (GSH) and glucuronate by cellular detoxifying enzymes [42]. Mammalian MRP1 also transports known immunomodulating agents such as leukotriene C4 (LTC4) with high affinity [11,43]. Only limited studies of schistosome or platyhelminth MRP1 have been carried out to date, and include the localization of a putative fluorescent substrate of mammalian MRP1 to the schistosome excretory system [44], and anti-MRP1 immunoreactivity along the tegumental cell layer in *Fasciola gigantica* [18].

Here, we examine the relationship between PZQ and SmMRP1, a schistosome homolog of mammalian MRP1. We show that, like SMDR2, SmMRP1 is transiently upregulated in adult schistosomes exposed to low concentrations of PZQ, though with a pattern somewhat different than SMDR2. Unlike SMDR2, SmMRP1 RNA is expressed at higher levels in males than in females. SmMRP1 also appears to have different distributions in the two sexes, possibly indicating distinct gender-specific roles. Furthermore, both SmMRP1 and SMDR2 RNAs are expressed at higher levels in PZQ-insensitive juvenile worms compared to PZQ-sensitive adults. These results add further support to the notion that schistosome MDR transporters may be playing important roles in the parasite's response to PZQ.

2. Materials and methods

2.1. Reagents

Praziquantel (Sigma) was dissolved in dimethyl sulfoxide for stock solutions, which were subsequently diluted to an appropriate concentration in culture media. The mouse monoclonal antibody against MRP1 was from Abcam (ab3371; MRPm6). The anti-rabbit tubulin antibody was from Santa Cruz Biotechnology (H-235).

2.2. Isolation and treatment of adult schistosomes

Female Swiss Webster mice infected with *S. mansoni* (NMRI strain) were obtained from the NIAID Schistosomiasis Resource Center at the Biomedical Research Institute in Rockville, MD. Mature adult (6–7 weeks post-infection) and juvenile (3–4 weeks post-infection) *S. mansoni* were collected by perfusion, as described [45], and maintained in RPMI (Invitrogen) plus 10% FBS (Sigma) and 1% penicillin/streptomycin at 37 °C and 5% CO_2. Following an overnight incubation, worms were either exposed to different concentrations of PZQ, or carrier (DMSO) for different time periods, either as pairs, or as separated male and female groups. Following incubation, worms were quick-frozen in liquid nitrogen and stored at −80 °C until further use.

2.3. RNA and protein extractions

Total RNA was extracted using either RNAqueous-4-PCR (Ambion), NucleoSpin RNA XS (Macherey-Nagel), or Trizol (Invitrogen) for quick-frozen cercariae and subsequently treated with Turbo-DNAase (Ambion) or rDNAase (Macherey-Nagel) according to the manufacturer's instructions. For protein extractions, worms were homogenized in cell disruption buffer (Ambion Paris Kit) with a cocktail of protease inhibitors (Sigma) at 4 °C and incubated for 15 min on ice. Lysates were centrifuged at 13,000 rpm for 10 min at 4 °C and the supernatant collected was used immediately or stored at −20 °C.

2.4. Real-time RT-PCR

Real-time RT-PCR was performed using the Brilliant II SYBR Green QRT-PCR Master Mix, 1-Step kit (Stratagene) on an Applied Biosystems 3500 instrument, according to the manufacturer's recommendations. For all sequences, the antisense PCR primer was used to prime first-strand synthesis. Following optimization experiments, either 50 or 75 ng of RNA was used for each reaction. Following incubation at 50 °C for 30 min for cDNA synthesis, reactions were denatured at 95 °C for 10 min, and a three-step cycle used for amplification (45 cycles at 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 30 s). Primers used for the amplification of SmMRP1 and 18S ribosomal RNA were SmMRP1 F (5′-GGTCGTACTGGTTCGGGTAA-3′) and SmMRP1 R (5′-TGAAACGTAACGTGCCAGAG-3′), and Sm18S F (5′-AGGAATTGACGGAAGGGCAC-3′) and Sm18S R (5′-ACCACCCACCGAATCAAGAAAG-3′), respectively. Primer pairs used for the amplification of SMDR2 were SMDR2-TM-F1 (5′-TCTGACAATCGACCTGGTG-3′) and SMDR2-TM-R1 (5′-CCAAGGAAGCAATGACTAAAAC-3′). Data were analyzed using the $2^{-\Delta\Delta}C_t$ method [46] to determine the relative expression ratio between target (SmMRP1, SMDR2) and reference gene (18S RNA). Using *S. mansoni* glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the reference gene, as described in [20], produced essentially identical results (data not shown).

2.5. Cloning of SmMRP1 cDNA

Total RNA was isolated from adult *S. mansoni* and single-stranded cDNA was synthesized as described [39]. The full-length coding region of SmMRP1 (NCBI Accession No. GU967672) was amplified using high-fidelity Phusion DNA polymerase (New England Biolabs) with primers (forward 5′-ATGACTTCCTGATGGACTATGTTTGTC-3′; reverse 5′-TCAATCAACAATATGAGCATCTTTAGCTA-3′) designed against the ends of the full-length coding region. The PCR conditions used included an initial denaturation at 98 °C for 30 s, followed by 35 cycles of amplification at 98 °C for 10 s, 50 °C for 30 s and 72 °C for 180 s with a final extension 72 °C for 10 min. The resultant 5.2 kb fragment was cloned initially into the pJAZZ-OK blunt vector using the BigEasy long PCR cloning kit (Lucigen) according to the manufacturer's recommendations, and sequenced. For further studies, SmMRP1 was subcloned into the NotI site of the mammalian expression vectors pXOOM [47] and pcDNA3.1 (Invitrogen).