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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 antischistosomal. 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.

Keywords

Schistosoma mansoni; multidrug resistance-associated protein; multidrug resistance; praziquantel; ABC transporter; ABCC1

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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 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 *S. 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 IC₅₀ of 17.4 μ M, 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.

MATERIALS AND METHODS

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).

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% CO₂. 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.

RNA and protein extractions

Total RNA was extracted using either RNeasy-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.

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 75ng 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 30s, 54°C for 30s, and 72°C for 30s). Primers used for the amplification of SmMRP1 and 18S ribosomal RNA were SmMRP1 F (5' GGTCGTAAGTTCGGGTAA 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).

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 10s, 50°C for 30s and 72°C for 180s with a final extension 72°C for 10 min. The resultant 5.2kb 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 Not I site of the mammalian expression vectors pXOOM [47] and pcDNA3.1 (Invitrogen).

In vitro protein expression

For *in vitro* protein expression, we tested two different templates. Construct A was the full-length coding sequence of SmMRP1 in pXOOM [47], and Construct B was full-length SmMRP1 in pcDNA3.1 (Invitrogen). A control reaction consisted of no added DNA. The templates were incubated with the Human *in vitro* Protein Expression Kit (Thermo Scientific Pierce) for 6 hours at 30°C according to the manufacturer's instructions. Translation products were analyzed by western blot as described below.

Western blot

Total protein concentrations were measured using a Bradford assay (Fermentas) with BSA (Sigma) as a standard. Subsequently, 25 µg of protein samples or *in vitro* expression reactions were loaded onto NuPage 4–12 % Bis-Tris gradient gels (Invitrogen) and resolved using MOPS buffer (Invitrogen), and transferred to a nitrocellulose membrane using an Xcell II™ Blot Module (Invitrogen). The membrane was blocked in blocking buffer [5% dry milk dissolved in Tris-buffered saline containing 0.5% Tween 20 (TBST)] at either 4°C overnight or room temperature for 3 h. Blots were incubated with mouse monoclonal anti-MRP1 antibody (1:250) or anti-rabbit β-tubulin (1:200) for 2 h at room temperature and washed 3 times in TBST. Blots were then incubated in either peroxidase-conjugated goat anti-mouse IgG (1:10,000; Jackson ImmunoResearch) or goat anti-rabbit IgG (1:10,000; Jackson ImmunoResearch), washed, and visualized with Supersignal West Pico Chemiluminescent Substrate (Thermo Scientific Pierce) according to the manufacturer's instructions.

Immunostaining

Adult *S. mansoni* (NMRI strain) were fixed in 10% neutral-buffered formalin (Thermo Scientific), embedded in paraffin sections and sliced into 6 µm sections with a motorized microtome (Leica RM2155). Slides were dewaxed in xylene for 10 min and immersed in a sequence of ethanol (95%, 90%, 80%, 70%) for 2 min each at room temperature. After hydration, slides were rinsed with 1× PBS and blocked with protein blocking agent

(StartingBlock™T20 PBS Blocking Buffer, Thermo Scientific) for 30 min at room temperature. Tissue sections were incubated with anti-mouse MRP1 (1:500) or without antibody (control) overnight at 4°C. After washing several times with 1× PBS, slides were incubated with fluorescent secondary Cy5-affinipure goat anti-mouse antibody (1:500 dilution, Jackson ImmunoResearch) for 30 min at room temperature. Following several washes with 1× PBS, slides were examined using a Nikon E600 microscope. Imaging was done using a SPOT digital camera and analyzed with SPOT Advance Software (Diagnostic Instruments), Photoshop 7.0 (Adobe), or Canvas (ACD Systems).

Statistics

Data are expressed as mean ± SD, and were tested for statistical significance using unpaired, two-tailed t-tests.

RESULTS

Adult *S. mansoni* express SmMRP1, a cDNA homologous to mammalian multidrug resistance-associated protein 1 (MRP1)

The *S. mansoni* genome database contains a highly significant match to mammalian MRP1 (Smp_171740), which we have named SmMRP1. Based on this sequence, we designed primers to amplify the entire coding region of SmMRP1 by RT-PCR from adult total RNA, and cloned it initially into the pJAZZ-OK vector. These inserts were subsequently subcloned into the pcDNA3.1 and pXOOM [47] vectors. The PCR product we amplified predicts an open reading frame with a molecular mass of ~210 kDa. Comparative sequence analysis between the predicted Smp_171740 coding region and our clones (SmMRP1) reproducibly reveals 95% amino acid identity, with an additional 183bp DNA insert following bp 42 in our cDNA. This results in the insertion of a unique stretch of 61 amino acids near the N-terminus of the SmMRP1 protein that is not found in mammalian MRP1 sequences. A second difference includes an insert and changed amino acid sequence near the C-terminus, between amino acid residues 1160 and 1245. Both of these stretches are found within the SmMRP1 gene (scaffold Smp_scaff000426), in regions that had been predicted to be introns. Overall, SmMRP1 shares 35% amino acid identity with mouse MRP1 (acc. no. **O35379**), with long stretches of nearly 100% identity separated by stretches of lower similarity. The N-terminal insert appears to be one of several regions in the SmMRP1 not found in other MRP1 proteins, particularly within the N-terminal region. The other insert does not appear to alter the level of amino acid identity to mouse MRP1 in that region of the protein.

In order to confirm the difference between the predicted open reading frame in the genome database and our cDNA sequence, we designed primers upstream and downstream of the extra 183bp DNA fragment and performed one-step RT-PCR from a different sample of adult total RNA. We also performed RT-PCR with 5' and 3' primers designed to amplify the entire open reading frame, and sequenced these PCR products without cloning. In both cases, we obtained the same sequence as we found in our SmMRP1 clones. Based on this information, we believe that the SmMRP1 sequence we obtained (NCBI accession no. **GU967672**) is in fact expressed in *S. mansoni* adults.

We employed a human cell-free expression system to test whether a mouse monoclonal antibody against human MRP1 (MRPm6) cross-reacts with recombinant SmMRP1. This antibody targets a conserved, 170 amino acid segment corresponding to the carboxy-terminal end and part of the carboxy-proximal nucleotide-binding domain of the human MRP1 protein [48]. This region of human MRP1 shows 55% amino acid identity to the corresponding region of SmMRP1, including several fully conserved stretches. We tested two different templates: a circular pXOOM vector [47] containing the full-length SmMRP1 coding sequence (Construct

A); and a circular pcDNA3.1 vector containing the full-length SmMRP1 (Construct B). After coupled transcription/translation, the products were resolved by gel electrophoresis, blotted, and probed with the anti-MRP1 monoclonal antibody. As shown in Fig. 1, immunoblot analysis detects a cross-reacting protein of ~200 kDa, but no comparable band is seen in samples incubated without DNA (No DNA Control).

Taken together, these data show that SmMRP1 can be expressed in a heterologous system and that it cross-reacts with this anti-MRP1 antibody.

SmMRP1 is expressed differentially in males and females

Expression of RNA encoding *S. mansoni* Pgp (SMDR2) has been shown to be higher in adult females than in males [38]. To determine the level of SmMRP1 RNA in the different *S. mansoni* sexes, we isolated total RNA from separated adult male and female worms and performed RT-PCR. In contrast to SMDR2, SmMRP1 RNA is expressed at 3.27 ± 1.68 -fold higher levels in males than in females (Fig. 2).

We also compared the distribution of anti-MRP1 immunoreactivity in males and females, using immunolocalization with anti-MRP1. Adult male and female worms were labeled with anti-MRP1 antibody, followed by Cy5-labeled secondary antibody (red), and visualized by immunofluorescence microscopy along with the nuclear counter stain DAPI (blue). As shown in Fig. 3, male worms show prominent immunofluorescent labeling around the testes and seminal vesicles (Fig. 3B, arrow) and in the epithelial cell layer surrounding the gut (Fig. 3C, arrow). In contrast, immunoreactivity in adult female worms is found towards the excretory pore (Fig. 3E, arrow) and in the subtegumental region (Fig. 3F, arrow). Worms incubated with only secondary Cy5-goat-anti-mouse IgG showed no detectable immunoreactive red fluorescence (Fig. 3A, D).

SmMRP1 and SMDR2 RNAs are expressed at higher levels in PZQ-insensitive juvenile schistosomes

Juvenile (4-week post-infection) *S. mansoni* are significantly less sensitive to PZQ than egg-laying adults [30–32,49]. We examined the expression levels of both *S. mansoni* SMDR2 (Pgp) and SmMRP1 RNA in juvenile worms that were 3–4 weeks post-infection. As shown in Fig. 4, juvenile worms show significantly higher expression of both SMDR2 and SmMRP1 RNAs than do egg-laying, PZQ-susceptible adults (7–8 weeks post-infection). Specifically, 3–4 week old worms express 2.41 ± 1.13 -fold higher SMDR2 RNA (Fig. 4A), and 2.46 ± 0.77 -fold higher SmMRP1 RNA (Fig. 4B) than do mature adults. Cercariae, which cease swimming and shed their tails in response to PZQ [50], show no significant differences in levels of SmMRP1 and SMDR2 RNA compared to mature adults (Fig. 4).

Levels of SmMRP1 RNA increase transiently in adult worms in response to PZQ

To test the effect of PZQ on SmMRP1 expression, adult worm pairs or separated male and female adult worms were chronically exposed in culture to a low, sub-lethal concentration of PZQ for different amounts of time. Total RNA was extracted from PZQ-exposed and control parasites, and used as template for qRT-PCR. The mixed-sex worms show a significant 2.68 ± 0.37 -fold increase in SmMRP1 expression at 6 h following exposure to 100 nM PZQ, and a 3.1 ± 1.2 -fold following exposure to 300 nM PZQ (Fig. 5A). Increases are also apparent at 3 h, but peak at 6 h, and by 24 h, SmMRP1 mRNA levels have returned to baseline (data not shown). Separated male worms express significantly higher (1.76 ± 0.87 -fold) SmMRP1 RNA (Fig. 5B), but females show only a non-significant 1.35 ± 0.47 -fold change following incubation in 100 nM PZQ for 6 h (Fig. 5C). The change in SmMRP1 RNA in the separated worms is less pronounced than in mixed-sex worms, perhaps indicating that an interaction between paired worms is required for a full response to PZQ. Control, untreated worms do not

show any increase through 24 h (data not shown). These data, combined with our previous results [20], show that both SMDR2 and SmMRP1, the two *S. mansoni* MDR transporters that have been tested, are upregulated in response to the antischistosomal drug PZQ.

Levels of an anti-MRP immunoreactive protein increase in adult schistosomes exposed to PZQ

To further characterize the modulation of SmMRP1 by PZQ, we examined the relative levels of SmMRP1 protein (or a cross-reacting MRP1-like protein species) in adult schistosomes in response to sub-lethal concentrations of PZQ (100–300 nM). Following 24 h of PZQ exposure of paired adult *S. mansoni*, an anti-MRP1 immunoreactive, ~200kDa band increases in at both concentrations (Fig. 6A, upper panel). There was no significant change in the β -tubulin loading control (Fig. 6A, lower panel). Similar to paired worms, PZQ-exposed male schistosomes also show an increase of the anti-MRP1-immunoreactive band at both concentrations (Fig. 6B, upper left), without significant change in the β -tubulin loading control (Fig. 6B, lower left). We also sometimes see evidence for an increase in band intensity in females as well, though the change is not as clear as in males (Fig. 6B, upper right). No detectable changes in expression levels of anti-MRP1 immunoreactivity are observed in *S. mansoni* exposed to PZQ for either 6 h or 12 h (data not shown).

DISCUSSION

MDR transporters are members of the ABC superfamily that function to remove toxic and xenobiotic compounds from cells. They were initially identified as underlying the phenomenon of multidrug resistance in mammalian tumor cells that developed cross-resistance to a broad range of unrelated drugs. They are also associated with drug resistance in helminths [25]. The first identified multidrug transporter in mammals was Pgp. Subsequently, other multidrug resistance transporters were discovered, including the MRPs. Here, we have shown that SmMRP1, a *S. mansoni* homolog of mammalian MRP1, is, like the Pgp homolog SMDR2, upregulated in worms following exposure to sub-lethal concentrations of PZQ. Furthermore, both SMDR2 and SmMRP1 are expressed at significantly higher levels in juvenile worms, which are PZQ-insensitive. Taken together with our results showing direct interaction of PZQ with SMDR2 [39], and increased SMDR2 expression in a schistosome isolate with reduced PZQ susceptibility [20], this body of evidence points to an association of higher MDR expression with reduced PZQ sensitivity, and suggests mechanisms by which these transporters could possibly mediate or maintain PZQ resistance. Nonetheless, we do not suggest that MDR transporters are primary molecular targets of PZQ. Rather, their increased expression, which occurs on a time scale of hours, may represent a mechanism to limit long-term downstream effects of the drug, including lethality. PZQ-induced worm lethality *in vitro* is not immediate, and can take days. It is possible that PZQ or biotransformed derivatives are inhibiting or, as substrates, overwhelming the schistosome MDR efflux transporters, either of which might serve as a signal for cells to increase transporter expression.

In mammals, the mechanism by which this type of signal is transduced is extremely complex, with upregulation seemingly dependent on different redundant signaling pathways related to cellular stress responses [51,52]. These pathways are only partially defined, but include MAP kinase, PI3 kinase, NF- κ B, and protein kinase C (PKC), and exhibit significant crosstalk, adding even further complexity. Whether schistosomes exploit similar or divergent mechanisms should prove to be an intriguing question for further investigation.

Mammalian MRP1 is one of 9 members of the human MRP family. Though they are within the same superfamily of ABC transporters, and have related functions, mammalian Pgp and MRPs typically share only 10–15% amino acid identity; similarly, SMDR2 and SmMRP1 share 13.5% amino acid identity. Though Pgp and MRP1 share some common substrates, MRP1 has

preference for anionic compounds, including compounds that have been conjugated to GSH and glucuronate by cellular detoxifying enzymes [53]. Interestingly, MRP1 and other ABC transporters appear to be involved in the regulation of immune responses, and transport known immunomodulating agents [11]. For example, leukotriene C4 (LTC4) is a high-affinity, endogenous GSH S-conjugate substrate for MRP1. In MRP1^{-/-} mice, leukotriene release from mast cells is reduced, as is their response to an inflammatory stimulus [54]. MRP1 has also been implicated in T-cell activation [55]. The possibility that schistosome MDRs such as SmMRP1 could be interacting with host immunomodulating agents or mediating the release of parasite modifiers of host responses warrants further investigation.

The significance of the higher levels of MDR transporters in juvenile worms (SmMRP1, SMDR2) and in a schistosome isolate with reduced PZQ susceptibility (SMDR2) remains to be determined. Increased levels of these transporters might play a role in the mechanism by which reduced PZQ susceptibility develops or is maintained, or could simply be a correlate of this process. In either case, if MDR upregulation proves to be a consistent feature found in worms with decreased PZQ sensitivity, it might at the least prove useful for development of markers for monitoring emergence of PZQ resistance. Interestingly, PZQ analogs with activity against juvenile worms have recently been described [56], and these compounds may prove to be useful tools in investigating these question.

In current models of Pgp efflux, the transporter binds substrate within the lipid bilayer of the cell membrane and exports the compound before it enters the cell [57]. Thus, upregulation of a Pgp transporter such as SMDR2, which we previously showed is inhibited by PZQ and transports BODIPY-PZQ [39], could effectively prevent a hydrophobic compound such as PZQ from ever reaching an intracellular target. Unlike Pgp, however, MRP1 is selective for substrates that have been biotransformed by intracellular detoxifying enzymes. There is essentially no information on the metabolism of PZQ within schistosome cells. It will be interesting to determine how PZQ is dealt with by schistosome cells and whether PZQ itself, or a metabolic derivative of PZQ, is a substrate for SmMRP1. If so, inhibition of SmMRP1 or other schistosome MDRs might be able to potentiate the effectiveness of PZQ. Furthermore, export of intracellular GSH via MRP1 is a marker of apoptosis, and GSH depletion likely plays an important signaling role [58,59]. Enhancement of this intracellular GSH depletion in the parasite via increased SmMRP1-mediated drug transport could represent a strategy to accelerate and dysregulate parasite apoptosis [23]. Similar approaches might also prove useful for attenuating PZQ resistance.

List of abbreviations

MDR	Multidrug resistance
MRP	Multidrug resistance-associated protein
PZQ	Praziquantel
Pgp	P-glycoprotein
ABC	ATP-binding-cassette

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Figure 1. A monoclonal anti-mouse MRP1 antibody cross reacts with SmMRP1 protein expressed *in vitro*

To check the cross reactivity of anti-mouse MRP1 antibody, circular pXOOM (Construct A) or pcDNA3.1 (Construct B) containing the full-length coding region of SmMRP1 was used as a template for *in vitro* protein expression. The samples were resolved and probed with anti-MRP1 antibody, which, detects a cross-reacting, ~200 kDa band (arrow) in the Construct A and Construct B lanes. Control samples incubated with no DNA (No DNA Control) did not show this band..

Figure 2. Male adults express higher levels of SmMRP1 RNA than females

Real-time RT-PCR analysis (n=6) of SmMRP1 from female (black bar) or male worms (grey bar) demonstrates approximately 3-fold higher expression of SmMRP1 in male worms. ** indicates $P \leq 0.01$, unpaired t-test.

Figure 3. Immunofluorescence localization of anti-MRP1 in *S. mansoni* adults

Adult male (upper panel) or female worms (lower panel) were fixed in 10% neutral buffered formalin, sectioned, and probed with (B, C, E and F) or without (A, D) anti-MRP1 antibody. Subsequent incubation with secondary Cy5-goat anti mouse IgG reveals immunoreactive red fluorescence around the testes (arrow, B) and epithelial cell layer surrounding the gut (arrow, C) in males. Female worms exhibit localized immunofluorescence towards the excretory pore (arrow, E) and sub-tegument (arrow, F), whereas control worms (A, D) incubated only with secondary Cy5 antibody did not show immunoreactivity. DAPI staining (blue) was used to counterstain nuclei.

Figure 4. PZQ-insensitive juvenile worms express higher levels of both SmMRP1 and SMDR2 RNA compared to PZQ-sensitive adult worms

A) Relative SmMRP1 RNA expression in *S. mansoni* mature (6–7 weeks post-infection) paired adults (black bar; n = 4), juveniles (3–4 weeks post-infection; grey bar; n = 4), or cercariae (white bar; n = 3). B) Relative SMDR2 expression (n=4) in *S. mansoni* mature (6–7 weeks post-infection) paired adults (black bar; n = 4), juveniles (3–4 weeks post-infection; grey bar; n = 4), or cercariae (white bar; n = 3).. All worms were incubated in RPMI for 24 h. For both SmMRP1 and SMDR2, 18S RNA was used as a reference to determine relative expression. * and ** indicate significant differences of $P \leq 0.05$ and $P \leq 0.01$, unpaired t-test, respectively.

Figure 5. Expression of SmMRP1 RNA is up regulated in response to PZQ

A) Relative expression of SmMRP1 RNA in adult *S. mansoni* pairs in the absence (black bar) or presence (white bars) of 100 nM (n = 5) or 300 nM (n = 4) PZQ. B) Increased SmMRP1 RNA expression in male (n=6) or female (C) worms (n=5) following PZQ exposure (100 nM). The fold changes in SmMRP1 RNA level were determined by quantitative RT-PCR using 18S RNA as the reference gene. * and ** indicate significant differences of $P \leq 0.05$ and $P \leq 0.01$, unpaired t-test, respectively.

Figure 6. Exposure of adult *S. mansoni* to PZQ results in increased expression of anti-MRP1 immunoreactive protein

A) Upregulation of MRP1-immunoreactive protein in adult *S. mansoni* pairs following PZQ (100, 300 nM; lanes 2, 3) or DMSO (0 nM, lane 1) exposure for 24h. The lower panel shows the loading control probed with an anti β -tubulin antibody. B) Increased expression of anti-MRP immunoreactive protein in male (upper left) or female (upper right) worms in response to different concentrations (100, 300n M) of PZQ for 24 h. There is no significant change in the loading control β -tubulin (lower panel). PZQ upregulates the expression of anti-MRP1 immunoreactive protein in males at both concentrations of PZQ.