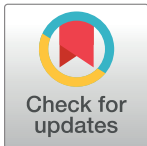REVIEW

Progress interrogating TRPM$_{PZQ}$ as the target of praziquantel

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Abstract

The drug praziquantel (PZQ) has served as the long-standing drug therapy for treatment of infections caused by parasitic flatworms. These encompass diseases caused by parasitic blood, lung, and liver flukes, as well as various tapeworm infections. Despite a history of clinical usage spanning over 4 decades, the parasite target of PZQ has long resisted identification. However, a flatworm transient receptor potential ion channel from the melastatin subfamily (TRPM$_{PZQ}$) was recently identified as a target for PZQ action. Here, recent experimental progress interrogating TRPM$_{PZQ}$ is evaluated, encompassing biochemical, pharmacological, genetic, and comparative phylogenetic data that highlight the properties of this ion channel. Various lines of evidence that support TRPM$_{PZQ}$ being the therapeutic target of PZQ are presented, together with additional priorities for further research into the mechanism of action of this important clinical drug.

Introduction

The drug praziquantel (PZQ) has served for decades as the key clinical agent for treating diseases caused by parasitic flatworms. Effective against the majority of these infections [1,2], it is recognized as one of 100 essential medications by the World Health Organization [3]. As a cheap, safe, broadly active, and well-scrutinized clinical therapy, PZQ has served as the keystone of mass drug administration campaigns to decrease the intensity and prevalence of schistosome infections in countries where schistosomiasis is endemic.

   PZQ is, however, an old drug. The anthelmintic activity of PZQ was first realized during a screening collaboration between Merck KGaA and Bayer AG in the 1970s [1,4,5]. Profiling a series of acylated pyrazinoisoquinoline-like compounds revealed the potent activity of PZQ against various trematode and cestodes. Three effects—(i) rapid cellular and tissue depolarization; (ii) a sustained muscle contraction causing worm paralysis; and (iii) damage to the worm tegument apparent as surface "blebbing"—serve as the cardinal triad of features caused by PZQ in all parasitic flatworms where PZQ displays efficacy (**Fig 1**). For each of these effects, the (R)-enantiomer of PZQ ((R)-PZQ) acted at lower concentrations than the (S)-enantiomer ((S)-PZQ), evidencing a preference for (R)-PZQ at the parasite target. Unfortunately, the identity of this target has resisted definition throughout subsequent decades of clinical usage, placing PZQ within a small minority of FDA-approved drugs with no elaborated molecular target [6].
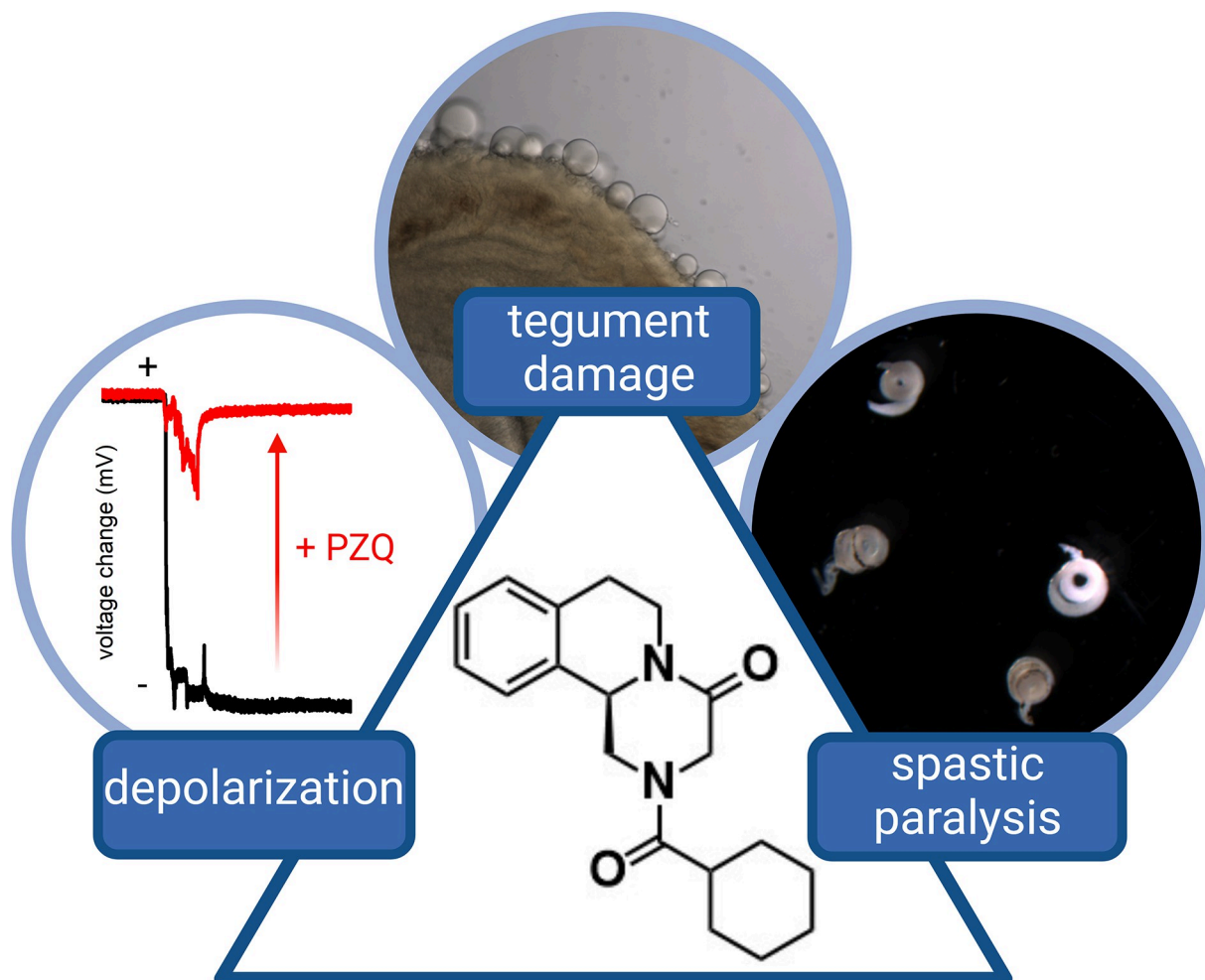
Fig 1. Cardinal effects of PZQ on schistosomes. PZQ treatment of schistosomes is associated with a triad of phenotypic effects: a rapid depolarization of muscle cells, a sustained spastic paralysis of the worm, and broad damage to the tegument manifest as surface blebbing and vesicularization. These effects are most obvious with the (*R*)-enantiomer of PZQ (center). Data are reproduced with permission from [23].

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Nevertheless, PZQ has proved a very effective drug in the clinic. However, opportunities for improvement certainly remain. These include optimization of formulations or derivatives that address the low oral bioavailability and rapid host metabolism of PZQ [7,8], as well as mitigation of other challenges (for example, bitter taste [9]) that result in poor compliance in the field [10]. Further opportunities relate to the lower efficacy of PZQ against certain parasites and life cycle stages—most clearly exemplified by the lack of PZQ activity against *Fasciola* species as well as the poor effectiveness of PZQ against juvenile schistosomes. Our understanding of why PZQ efficacy varies in these situations has been hampered by our lack of knowledge of the molecular target of PZQ. This has long proved a frustrating roadblock. Knowledge of the target would catalyze a better understanding of endogenous signaling pathways essential for parasite viability and thereby vulnerabilities to chemotherapeutic attack. This would also enable target-based drug screening efforts to catalyze discovery of new anthelmintics. Finally, a validated target would enable prospective surveillance for sequence variation, occurring naturally or in response to drug pressure, which could be one of many mechanisms that underpin decreased PZQ effectiveness in the field [11].

For all these reasons, the recent identification of a parasite target for PZQ is a significant breakthrough [12]. This target is a parasite ion channel from the transient receptor potential melastatin family, named TRPM$_{PZQ}$ [12]. TRPM$_{PZQ}$ has been prioritized as an appealing target as it displays properties consistent with the known action of PZQ against parasitic flatworms. The purpose of this review is to summarize experimental evidence collated since the discovery of TRPM$_{PZQ}$ [12,13] that has interrogated the candidature of this ion channel as the clinically relevant target of PZQ. Efforts have focused on understanding (i) the key properties of TRPM$_{PZQ}$; (ii) the impact of variation in TRPM$_{PZQ}$ sequence and expression; and (iii) insight from novel pharmacological tools. Ten pieces of evidence supporting correct target validation of TRPM$_{PZQ}$ are presented, concluding with a discussion of caveats and some future priorities for investigation.

Key properties of *Sm*.TRPM$_{PZQ}$

In 2019, Park and colleagues identified a TRP channel from *Schistosoma mansoni* (named *Sm*. TRPM$_{PZQ}$) which when heterologously expressed in mammalian cells mediated robust cellular Ca^{2+} signals on exposure to PZQ [12]. Consistent with the long-held focus on a "Ca^{2+} channel hypothesis" for PZQ action [13–15], effort to further investigate the properties of this Ca^{2+}-permeable ion channel target held merit.

1. The basic properties of TRPM$_{PZQ}$ replicate the characteristics of PZQ action on schistosomes. Enticingly, the attributes of the TRPM$_{PZQ}$ response to PZQ were consistent with the well-known features of PZQ action on schistosomes. First, the potency of PZQ at TRPM$_{PZQ}$ was in the hundreds of nanomolar range (EC$_{50}$ for (*R*)-PZQ was approximately 150 nM at 37°C), consistent with PZQ action on worms ex vivo [12]. Second, (*R*)-PZQ was more potent than (*S*)-PZQ, consistent with the recognized stereoselectivity of the enantiomers versus parasitic flatworms [12]. Third, the kinetics of activation of TRPM$_{PZQ}$ were rapid in onset with little apparent desensitization of the channel toward PZQ, consistent with the sustained profile of schistosome muscle contraction evoked by PZQ [16,17]. Fourth, the response of *S. mansoni* TRPM$_{PZQ}$ to PZQ was attenuated by Mg^{2+} and blocked by La^{3+}, consistent with the effects of these metal ions on *S. mansoni* muscle contractility [16,17]. Overall, the congruence between these basic characteristics of PZQ-evoked TRPM$_{PZQ}$ activation and worm responsivity to PZQ underscored the promising candidature of TRPM$_{PZQ}$ as the elusive parasitic target of PZQ [12,13].

2. An endogenous current activated by PZQ matches the biophysical signature of TRPM$_{PZQ}$. While identified on the basis of monitoring Ca^{2+} permeability, TRPM$_{PZQ}$ is a nonselective cation channel permeable to both monovalent and divalent cations [18]. Again, this is consistent with the ability of PZQ to stimulate the influx of Na$^+$ and Ca^{2+}, and the loss of K$^+$ from intact schistosomes [19]. Membrane depolarization consequent to TRPM$_{PZQ}$ activation can be resolved in Ca^{2+}-free solutions using a fluorescent membrane potential reporter [18] or by recording currents in Ca^{2+}-free media [18,20,21]. TRPM$_{PZQ}$ behaves as a voltage-independent ion channel based on a linear current–voltage relationship with a conductance of 110 to 130 pS for *S. mansoni*, *S. haematobium*, and *S. japonicum* TRPM$_{PZQ}$ (recorded in symmetrical 145 mM NaCl) and an open probability, P$_{open}$ = 0.4 to 0.6 [18,21]. These characteristics, combined with a lack of desensitization of channel opening toward PZQ, may endow TRPM$_{PZQ}$ with the ability to mediate a long-lasting depolarization in cell types where it is expressed [18].

However, native PZQ-evoked currents have never been recorded from any parasitic flatworm. This is likely due to the technical challenges of performing these measurements, but also because of our not knowing what exactly to look for and where best to look. Recent effort

to resolve the single-channel properties of TRPM$_{PZQ}$ in vitro have established a "biophysical signature" for Sm.TRPM$_{PZQ}$, defining a clear search algorithm as well as the optimal recording conditions to search for a native PZQ-evoked current [18]. Further, RNAseq datasets have revealed that TRPM$_{PZQ}$ is expressed in many excitable cells, present in multiple neuronal cell types [22].

These insights improved the feasibility of a new hunt to find an endogenous PZQ-evoked current. Chulkov and colleagues attempted such analyses using invasive electrophysiology to record currents from a live adult schistosome [23]. Despite the challenges of this approach, single-channel responses evoked by PZQ could be resolved from recordings made in "neuronal" tissues, including the anterior ganglia and main nerve cord of male worms [23]. In contrast, no response to PZQ was evident in recordings from "muscle" tissue, or PZQ-derived tegumental vesicles under similar conditions. The native PZQ-activated ion channel displayed properties (linear I-V, Cs$^+$ permeability, P$_{open}$, conductance) consistent with the properties of Sm.TRPM$_{PZQ}$ measured in vitro [18]. Further, the PZQ-evoked current was blocked by a Sm.TRPM$_{PZQ}$ antagonist [23]. That the properties of an endogenous PZQ-activated current in an adult schistosome closely match the characteristics of Sm.TRPM$_{PZQ}$ support correct target identification.

3. TRPM$_{PZQ}$ is present in all flatworms that show sensitivity to PZQ. TRPM$_{PZQ}$ must be present in all parasites that exhibit sensitivity to PZQ; otherwise, another target must exist to mediate PZQ action in these worms. Bioinformatic analyses have shown this to be the case. Scrutiny of available genomic and transcriptomic resources revealed the revealed the presence of TRPM$_{PZQ}$ orthologs in all available flatworm genomes [12,24]. TRPM$_{PZQ}$ orthologs from 11 of these different species have been functionally profiled in vitro. These encompass TRPM$_{PZQ}$ from schistosomes (*S. mansoni*, Sm.TRPM$_{PZQ}$; *S. japonicum* Sj.TRPM$_{PZQ}$; and *S. haematobium*, Sh.TRPM$_{PZQ}$), *Clonorchis sinensis* (*Cs*.TRPM$_{PZQ}$), *Opisthorchis viverrini* (*Ov*.TRPM$_{PZQ}$), *Echinostoma caproni* (*Ec*.TRPM$_{PZQ}$), *Fasciola* species (*F. hepatica*, Fh.TRPM$_{PZQ}$ and *F. gigantica*, Fg.TRPM$_{PZQ}$), tapeworms (*Echinococcus granulosus*, Eg.TRPM$_{PZQ}$ and *Mesocestoides corti*, Mc.TRPM$_{PZQ}$) as well as a free-living flatworm representative (*Macrostomum lignano*, Ml.TRPM$_{PZQ}$). All these orthologs, with the exception of TRPM$_{PZQ}$ from *Fasciola* species (*Fh*.TRPM$_{PZQ}$ and *Fg*.TRPM$_{PZQ}$) are sensitive to PZQ, with (*R*)-PZQ being the more active enantiomer in every case [24,25]. While not a comprehensive analysis, data from these functional profiling efforts to date remain consistent with the known clinical utility of PZQ for treating infections caused by these different parasitic flatworms. *Fasciola* infections are known to be refractory to PZQ treatment. Our understanding of the molecular basis for this insensitivity is discussed in the next section.

Genetic variation impacting TRPMPZQ function

Additional support for TRPM$_{PZQ}$ as the therapeutically relevant target of PZQ comes from the clear correlation between species- and strain-specific properties of TRPM$_{PZQ}$ and the overall sensitivity of these different parasitic flatworms to PZQ.

4. *Fasciola* TRPM$_{PZQ}$ provides a clear molecular explanation for the insensitivity of these liver flukes to PZQ. Liver flukes from the genus *Fasciola* are insensitive to PZQ and epsiprantel [2,26,27]. Human fascioliasis is refractory to treatment by PZQ [28,29]. An explanation for the lack of PZQ efficacy against these particular parasites has long been lacking. Suggestions have encompassed an impermeability of the liver fluke tegument to PZQ, efficient export of PZQ from *Fasciola*, or that the target of PZQ is absent in *Fasciola* spp. [26]. TRPM$_{PZQ}$ is, however, present in *Fasciola* spp. (see point #3), and functional analysis of TRPM$_{PZQ}$ provided a simple explanation for why PZQ does not work against these particular liver fluke infections.

Park and colleagues identified a single nucleotide variation between *Fasciola* TRPM$_{PZQ}$ and TRPM$_{PZQ}$ in other trematodes that yields an amino acid change within the binding pocket of *Fasciola* TRPM$_{PZQ}$, encoding a threonine residue instead of an asparagine residue (**Fig 2A**; [25]). This difference occurs at a critical position that is necessary for binding PZQ: The asparagine residue in transmembrane helix 1 (TM1) of schistosome TRPM$_{PZQ}$ is predicted to form a hydrogen bond with the internal carbonyl of PZQ [25] (**Fig 2B**). This interaction is predicted to be lacking between PZQ and *Fasciola* sp. TRPM$_{PZQ}$ (**Fig 2C**). When this residue was mutated to a threonine in *Sm*.TRPM$_{PZQ}$, PZQ could no longer activate the ion channel [25]. Reciprocally, mutation of the threonine residue to an asparagine within *Fasciola hepatica* or *Fasciola gigantica* TRPM$_{PZQ}$ realized a "gain-of-function," and PZQ became a potent activator of the *Fasciola* TRPM$_{PZQ}$ channel [24,25] (**Fig 2D**). Therefore, even though this variation represents a minimal and conservative amino acid replacement, the change in the TRPM$_{PZQ}$ binding pocket was sufficient to abrogate PZQ activity [24,25].

The selective pressures, if any, underpinning this change in *Fasciola* TRPM$_{PZQ}$ are unknown. Possibly, it may relate to the exposure to natural products during the *Fasciola* lifecycle (for example, compounds in watercress leaves where infective metacercariae are attached [30]) that could adversely activate TRPM$_{PZQ}$ in the absence of such this adaptation within the ligand binding pocket. Many phytochemicals act as TRP channel ligands [31].

Whatever the explanation, elucidation of the molecular basis of *Fasciola* TRPM$_{PZQ}$ insensitivity toward PZQ enabled a rational approach to develop new fasciocidal agents that are tolerant of this variation. Development of novel TRPM$_{PZQ}$ activators is currently a focus of ongoing investigation. One such chemotype—a benzamidoquinazolinone (BZQ)—which potently activated both *Sm*.TRPM$_{PZQ}$ and *Fh*.TRPM$_{PZQ}$ was recently identified [32]. The basis for this dual activation depends on a different binding conformation of BZQ within the TRPM$_{PZQ}$ VSLD binding pocket, such that the variant position on the S1 helix is not important for BZQ binding [32]. BZQ engages the S1 helix through a different interaction, conserved in both *Sm*.TRPM$_{PZQ}$ and *Fh*.TRPM$_{PZQ}$. Exposure of schistosomes to BZQ, like PZQ, caused a rapid and sustained contraction with obvious surface damage. Similarly, application of BZQ to *Fasciola hepatica* also caused a rapid, spastic contraction and tegumental damage. That a *Fasciola* TRPM$_{PZQ}$ activator identified by target-based screening is deleterious to liver fluke and phenocopies PZQ action on schistosomes further supports correct target validation of TRPM$_{PZQ}$.

This molecular insight should prompt wariness given the precedence this explanation establishes for the viability of a PZQ-insensitive TRPM$_{PZQ}$ channel maintained over evolutionary time. It would obviously be concerning if a similar TRPM$_{PZQ}$ variant was found naturally or occurred in response to the selective pressure associated with PZQ exposure during mass drug administrative campaigns. Analysis of TRPM$_{PZQ}$ sequence and/or expression levels as potential routes to PZQ resistance would be worthwhile in scenarios such as persistent "hotspots" after mass drug administration campaigns [33] or obvious losses of PZQ efficacy in veterinary [34,35] or aquaculture treatments [36,37]. Effort to catalogue TRPM$_{PZQ}$ variants, coupled with insight as to the functional consequences of such variation, will be crucial for surveillance of changes underpinning decreases in the clinical effectiveness of PZQ.

5. Schistosomes genetically selected for low PZQ sensitivity show low expression of TRPM$_{PZQ}$. A genome-wide association study using a mixed population of PZQ-resistant and PZQ-sensitive *S. mansoni* worms identified a 4MB region on chromosome 3, which harbored many genes at the highest association peak, including the gene encoding *Sm*.TRPMPZQ [38]. Marker-assisted selection using a single nucleotide polymorphism (SNP) present in the *Sm*.TRPMPZQ gene and associated with PZQ responsiveness allowed for the generation of 2 populations of schistosomes that displayed a remarkable >377-fold difference in PZQ
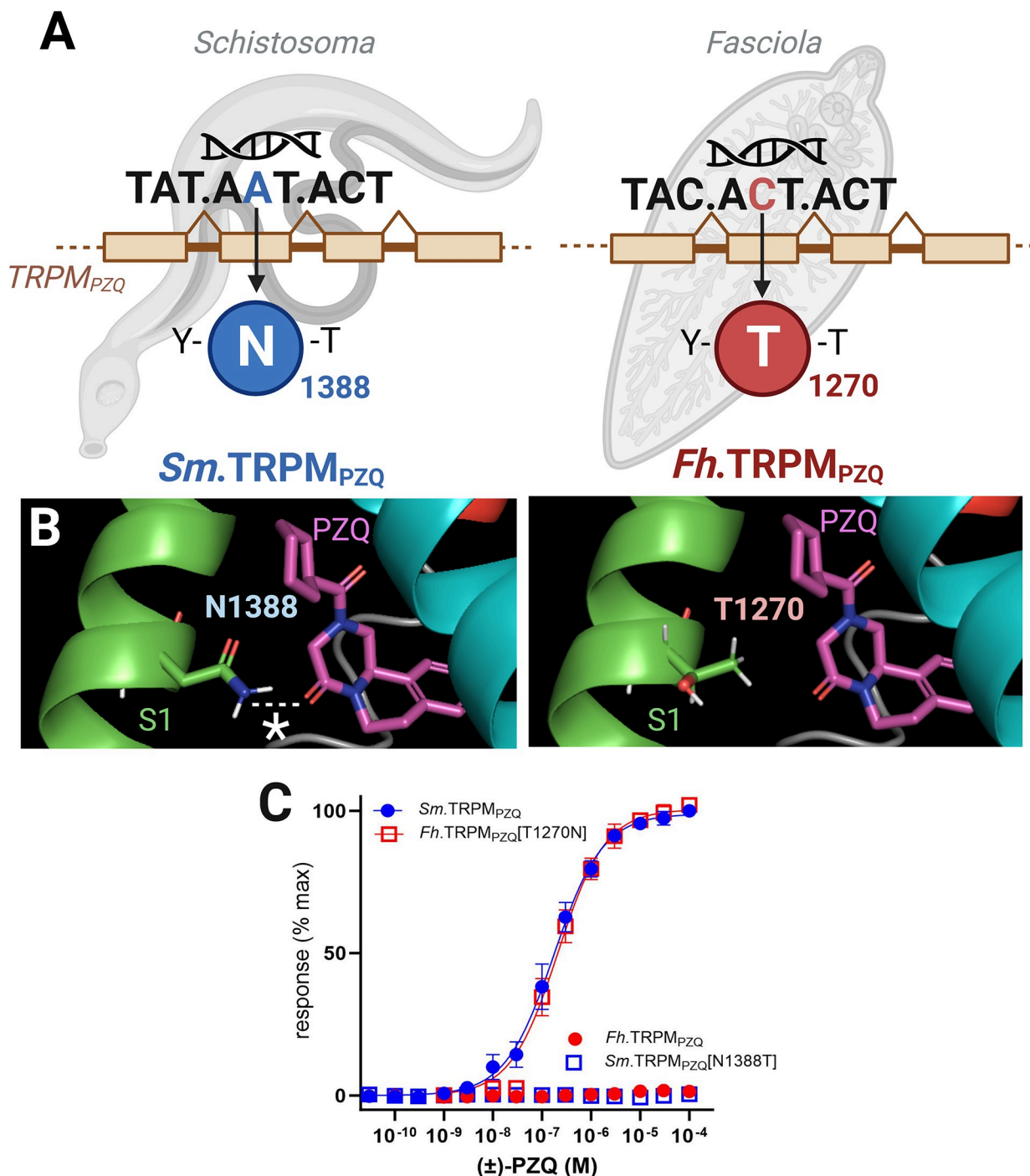
Fig 2. Genetic determinants of PZQ sensitivity. (**A**) A single nucleotide difference occurs at position 2 of the reading frame within an exon of the *TRPM$_{PZQ}$* gene that forms part of the PZQ binding pocket in *S. mansoni* TRPM$_{PZQ}$ (left) and *F. hepatica* TRPM$_{PZQ}$ (right). This results in different amino acids—asparagine in *S. mansoni* TRPM$_{PZQ}$ (N1388 in *Sm*.TRPM$_{PZQ}$) versus threonine in *F. hepatica* TRPM$_{PZQ}$ (T1270 in *Fh*. TRPM$_{PZQ}$)—at an equivalent position within the VSLD binding pocket in TRPM$_{PZQ}$ of these different flukes. This difference also occurs in *F. gigantica TRPM$_{PZQ}$*. (**B**) Location of this S1 helix reside (N1388 in *Sm*.TRPM$_{PZQ}$ versus T1270 in *Fh*.TRPM$_{PZQ}$) in a homology model *Sm*. TRPM$_{PZQ}$ relative to the predicted PZQ binding poise (magenta). The availability of N1388 to hydrogen bond with the internal carbonyl group of PZQ is inferred as important for PZQ activation of *Sm*.TRPM$_{PZQ}$. T1270 is either unavailable for hydrogen bonding, or this variation impacts binding pocket architecture in a manner deleterious to PZQ efficacy. (**C**) Concentration response curves comparing activation of wild-type *Sm*. TRPM$_{PZQ}$ and *Fh*.TRPM$_{PZQ}$ by PZQ (circles), as well as the effect of the reciprocal binding pocket mutants (*Sm*.TRPM[N1388T]$_{PZQ}$ and *Fh*. TRPM[T1270N]$_{PZQ}$, squares) on responsivity to (±)-PZQ. Data adapted from [25]. Panels in this Figure were created using BioRender.com.

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sensitivity. These populations were fixed for alternative alleles at a SNP genotyped within *Sm*. TRPM$_{PZQ}$, as well as 2 different proximal 150 kb deletions (one quite close to *Sm*.TRPM$_{PZQ}$ and one near a transcription factor). Of the genes within this locus, *Sm*.TRPM$_{PZQ}$ uniquely exhibited lower expression in adult male worms that displayed poor PZQ sensitivity [38]. This elegant body of work provides strong evidence that variation within, or near to, the *TRPM$_{PZQ}$* locus confers decreased responsivity to PZQ [38,39].

One explanation for this observation is that the lower sensitivity to PZQ results from lower levels of *Sm*.TRPM$_{PZQ}$ expression, which results in a less robust response or a more facile recovery of cells after PZQ exposure. That differential expression of *Sm*.TRPM$_{PZQ}$ may result in varied sensitivity to PZQ also chimes with the stronger response to PZQ in adult male versus female schistosomes [40,41], which correlates with the higher levels of *Sm*.TRPM$_{PZQ}$ expression in male worms [38,42]. *Sm*.TRPM$_{PZQ}$ shows lower expression in male or female worms that display low sensitivity to PZQ [38]. However, TRPM$_{PZQ}$ mRNA levels in juvenile schistosomes are equivalent or higher than those found in adults [38,42], so other explanations must underpin the lower sensitivity of immature worms to PZQ. Again, this may be caused by a less robust response (unique regulation of TRPM$_{PZQ}$ activity or ion channel expression in this specific lifecycle stage) or a more facile recovery to drug exposure (stronger tissue repair in juvenile worms). Overall, resolution of the regulatory mechanisms that control TRPM$_{PZQ}$ expression and correlating TRPM$_{PZQ}$ expression in parasitic flatworms with the effectiveness of PZQ treatment will be priorities for future study.

6. The differential sensitivity of various parasites to PZQ correlates with the differential PZQ sensitivity of TRPM$_{PZQ}$ orthologs in these different flatworms. The sensitivity of different parasitic flatworms toward PZQ is not the same; for example, while PZQ was originally recognized as effective against trematodes and cestodes [1,43], the sensitivity of cestodes to PZQ is highest. Sensitivity to PZQ is manifest at low nanomolar concentrations against some cestodes [44,45]. Blood flukes typically respond to PZQ in the hundreds of nanomolar range [2], and free-living flatworms show responses in the micromolar range [24]. *Fasciola* spp. represent an extreme example showing lack of sensitivity toward PZQ.

Efforts to measure the sensitivity of TRPM$_{PZQ}$ orthologs from representatives of each of these groups demonstrated that TRPM$_{PZQ}$ sensitivity to PZQ aligns well with the observed worm sensitivity to PZQ. Two cyclophyllidean cestode TRPM$_{PZQ}$ channels—*Mesocestoides corti* TRPM$_{PZQ}$ (*Mc*.TRPM$_{PZQ}$) and *Echinococcus granulosus* TRPM$_{PZQ}$ (*Eg*.TRPM$_{PZQ}$)—were potently activated by (*R*)-PZQ (EC$_{50}$ = 82 ± 3 nM for *Mc*.TRPM$_{PZQ}$, EC$_{50}$ = 55 ± 6 nM for *Eg*. TRPM$_{PZQ}$; [24]), consistent with the high sensitivity of this group of cestodes to PZQ. In contrast, TRPM$_{PZQ}$ from a free-living flatworm representative, *Macrostomum lignano* (*Ml*. TRPM$_{PZQ}$), was activated by (*R*)-PZQ with approximately 300-fold lower potency (EC$_{50}$ = 18 ± 0.8 μM; [24]), consistent with the concentration range of PZQ activity against free-living flatworms. Variation in the amino acids residues that line the orthosteric binding pocket of the different TRPM$_{PZQ}$ orthologs likely contributes to this differential sensitivity. Functionally impactful residues include (i) a histidine residue in cestode TRPM$_{PZQ}$ orthologs found at the same S1 helix position, which dictates the PZQ insensitivity of *Fasciola*, and (ii) an acidic amino acid residue found within the TRP helix. This latter residue, represented by an aspartic acid residue in the high sensitivity TRPM$_{PZQ}$ orthologs of trematodes and cyclophyllidean cestodes, is a glutamic acid residue in other parasitic TRPM$_{PZQ}$ orthologs and TRPM paralogs [24]. The presence of the glutamic acid variant confers lower sensitivity to PZQ in these channels, with application of molecular dynamics and metadynamic modelling methods suggesting the glutamic acid residue projects into the PZQ binding pocket ablating a critical receptor–ligand interaction required for high affinity PZQ binding [24]. Identification of this acidic "gatekeeper" residue provides an explanation for the lower observed clinical sensitivity to PZQ

in parasitic flatworm infections that harbor a glutamate at this position, while parasites that carry an aspartate at this position (trematodes and cyclophyllidean cestodes) uniquely display high sensitivity to PZQ [2,46]. The properties of TRPM$_{PZQ}$ orthologs from different flatworms, therefore, show a consistent correlation between worm sensitivity to PZQ and TRPM$_{PZQ}$ ortholog sensitivity to PZQ.

Pharmacology of TRPM$_{PZQ}$

With a candidate parasite target for PZQ identified, target-based drug screening approaches become feasible. Such efforts have realized additional insight into the properties of TRPM$_{PZQ}$.

7. The structural–activity relationship of drugs causing *Sm*.TRPM$_{PZQ}$ activation and worm contraction are similar. Functional profiling of a series of 43 PZQ analogs and non-obvious derivatives against *Sm*.TRPM$_{PZQ}$ ranked the potency of all these analogs in terms of TRPM$_{PZQ}$ activation [25]. The same analogs were then examined for their potency in causing spastic contraction of adult schistosome worms [25]. The structure–activity fingerprint for these analogs was almost identical in both assays. The "tightness" of the structure–activity relationship (SAR) of PZQ analogs at causing paralysis was also mirrored by the same strict SAR for efficacy at *Sm*.TRPM$_{PZQ}$. Such stringency around the pharmacophore of PZQ has long been recognized [1]. Retrospective analysis of data from over 250 PZQ analogs revealed only 4% of synthesized PZQ derivatives displayed equivalent activity to PZQ [47]. Such congruence between the SAR of the contractile response and the pharmacological profile of this ion channel in vitro is again consistent with *Sm*.TRPM$_{PZQ}$ acting as the mediator of PZQ action.

8. The different structure–activity relationship of TRPM$_{PZQ}$ between parasitic flatworms varies in line with worm sensitivity to different analogs. When the SAR of PZQ was elaborated [1], it became evident that particular PZQ analogs displayed differential activities against different types of parasite. For example, some PZQ analogs—3-pyridine analogs and certain modifications of the cyclohexyl ring—showed considerably greater activity toward cestodes than against schistosomes [1]. Is this differential bioactivity mirrored by different structure activity relationships at schistosome and cestode TRPM$_{PZQ}$? The answer is yes, with good alignment between analog activity against different parasites and the underlying selectivity of TRPM$_{PZQ}$ orthologs in these different species [48]. The SAR of TRPM$_{PZQ}$ in different parasites is, therefore, not identical. Differences in the amino acids lining the transmembrane TRPM$_{PZQ}$ ligand binding pocket likely underpin these effects, highlighting future potential for developing drugs tailored toward specific TRPM$_{PZQ}$ targets and problematic clinical infections that are more refractory toward PZQ treatment. That the distinct pharmacological signatures of parasites toward PZQ analogs mirrors the properties of TRPM$_{PZQ}$ in the different parasites further supports TRPM$_{PZQ}$ as the therapeutically relevant target.

9. Other agonists of TRPM$_{PZQ}$ phenocopy PZQ. If TRPM$_{PZQ}$ is the target of PZQ, other activators of TRPM$_{PZQ}$ should mimic PZQ action. If such TRPM$_{PZQ}$ activators do not phenocopy PZQ action, then PZQ must also engage other targets that contribute to the drug's therapeutic activity. To develop this line of enquiry, alternative activators of TRPM$_{PZQ}$ needed to be found. Chulkov and colleagues executed a target-based screen of approximately 16,000 compounds against *Sm*.TRPM$_{PZQ}$ using a miniaturized fluorescence reporter assay [49]. This screen resulted in the identification of a single TRPM$_{PZQ}$ agonist "hit" that surpassed triage criteria. This low hit rate (0.06%) in this screen was again consistent with the known stringent SAR of PZQ and TRPM$_{PZQ}$. The *Sm*.TRPM$_{PZQ}$ activator was named AG1 (<u>ag</u>onist-<u>1</u>) and was less potent than PZQ (EC$_{50}$ approximately 9 μM; [49]). Nevertheless, AG1 activated *Sm*.TRPM$_{PZQ}$ similarly to PZQ, eliciting non-desensitizing, ohmic currents when profiled electrophysiologically [49]. Further, a VSLD binding pocket mutant ablated both PZQ and AG1

activation of Sm.TRPM$_{PZQ}$, suggesting a similar action of both ligands through engagement of the transmembrane VSLD ligand binding site. While this does not prove that AG1 is selective for Sm.TRPM$_{PZQ}$, a novel Sm.TRPM$_{PZQ}$ activator was found from this screening effort. Notably, AG1 represented a different chemotype to PZQ. Whereas PZQ has a pyrazinoisoquinoline core, AG1 is a triazolopyridine derivative. Their common activation of TRPM$_{PZQ}$ highlighted the druggability of this novel target. Interestingly, AG1 was a known compound (MV688313, LSHTM-1945) previously identified as a high priority "hit" in a large, phenotypic screen (approximately 300,000 compounds) against different schistosome life cycle stages [50]. That a phenotypic screen and a target-based screen completed by independent groups converged on the same ligand, unmasked as a Sm.TRPM$_{PZQ}$ activator, provided further reassurance of correct target identification.

10. Antagonists of Sm.TRPM$_{PZQ}$ decrease parasite sensitivity toward PZQ. The same target-based screen against Sm.TRPM$_{PZQ}$ also yielded many potential blockers of this channel [49]. These candidate blockers have yet to be studied and appraised as to their mode of action, for example, whether they function as competitive inhibitors of PZQ within the same VSLD binding pocket, pore blockers, or noncompetitive inhibitors of the TRPM$_{PZQ}$ complex. Only the effects of one compound, ANT1 ("antagonist-1"), have been evaluated to date. ANT1, a substituted pyrazine, blocked the effects of PZQ measured in either a metabolic or a motility assay, such that ANT1 application recovered normal worm movement and viability in the presence of PZQ [38,49]. That a Sm.TRPM$_{PZQ}$ antagonist blocks the action of PZQ on schistosomes again supports the candidacy of TRPM$_{PZQ}$ as the relevant in vivo target of PZQ. ANT1 also blocked the native current evoked by PZQ in schistosomes [23]. The utility of TRPM$_{PZQ}$ blockers (as opposed to TRPM$_{PZQ}$ activators) as potential anthelmintics has not yet been explored. However, further study of these chemotypes is important as these efforts will provide useful tools for inhibiting the function of TRPM$_{PZQ}$ to unmask the endogenous role of this ion channel throughout the parasite lifecycle.

Caveats and future directions

Collectively, these 10 lines of evidence provide strong support for TRPM$_{PZQ}$ acting as the therapeutic target of PZQ, with the experimental data discussed above proving consistent with correct target validation. However, caution is always merited, and further questions remain. One wryly notes that even for cancer drugs undergoing clinical trials in humans, their assumed targets have often retrospectively been shown to be false [51,52]. This underscores the importance of coalescing multiple lines of evidence to underpin target validation [52,53]. In this regard, 3 areas merit further attention [13].

Validation through genetic loss of function analyses. Insight from functional genetic approaches is needed. Results from knockdown or knockout analyses, to ablate TRPM$_{PZQ}$ expression in parasites, have yet to be reported. Neither of these approaches are trivial to execute: Knockdown by RNA interference (RNAi) can be finicky depending on the target, how abundant it is and where it is expressed in the worm. Stable transgenesis in schistosomes is also an active focus for optimization. TRPM$_{PZQ}$ is not abundantly expressed at the surface of the worm but is found within excitable cells. The large cation flux mediated by TRPM$_{PZQ}$ would likely necessitate a highly penetrant knockdown of TRPM$_{PZQ}$ for RNAi data to be interpretable, as residual expression of TRPM$_{PZQ}$ could still support a robust depolarization response to PZQ. Challenges related to off-target effects with RNAi, and the adequacy of controls for many commonly scored phenotypes, also persist [54]. But provided TRPM$_{PZQ}$ is not crucial for parasite viability, these genetic loss-of-function approaches will provide critical insight as to the essentiality of TRPM$_{PZQ}$ for PZQ action. The availability of small molecule blockers of TRPM$_{PZQ}$ (see point #10) will complement these genetic loss-of-function

approaches as pharmacological blockade of TRPM$_{PZQ}$ should phenocopy and thereby validate RNAi effects. Clearly, if PZQ-evoked depolarization, contraction, and surface damage phenotypes persist in the absence of TRPM$_{PZQ}$, then other targets must mediate these effects. TRPM$_{PZQ}$, despite the aforementioned evidence, would then be a "false" target in relation to the anthelmintic activity of PZQ.

Other targets? PZQ inevitably has more than one target, consistent with the polypharmacological profile expected with any small molecule [55,56]. Many of these will be "secondary" targets, with these interactions not recapitulating the high sensitivity and stereoselectivity displayed by TRPM$_{PZQ}$ (the "primary" target). For example, in humans, where the process of target identification is more facile, PZQ has been shown to regulate multiple TRP channels [57,58] and several GPCRs [59]. However, these interactions exhibit lower sensitivities (micromolar at best) and often different stereochemistry (for example, hTRPM8 is only activated by (S)-PZQ [58]). For the human 5-HT$_{2B}$ receptor, where a (R)-PZQ binding pose has been defined and validated, the lower sensitivity of 5-HT$_{2B}$ toward PZQ (EC$_{50}$ in low micromolar range [59,60]) can be explained by the loss of specific binding interactions that been shown to anchor PZQ within the schistosome TRPM$_{PZQ}$ binding pocket. For example, whereas hydrogen-bonding interactions occur in TRPM$_{PZQ}$ to both the carbonyl groups of PZQ, only a single hydrogen-bond interaction is predicted in the human 5-HT$_{2B}$ binding pocket [60]. Loss of optimal hydrogen-bond interactions will decrease binding affinity [61], likely explaining the shift from the "hundreds of nanomolar" to the "micromolar" sensitivity range, even though "selective" binding (5-HT$_{2B}$ compared with 5-HT$_{2A}$ or 5-HT$_{2C}$) is still evident. These host targets may be relevant to several side effects associated with PZQ (5-HT$_{2B}$: smooth muscle contraction underpinning nausea, abdominal pains; TRPM8: poor taste), and, potentially, also therapeutic efficacy (vascular contraction in mesenteric vessels) by aiding the hepatic shift of contracted worms [59,60].

Just as with the discovery of such "secondary" targets in humans, secondary parasite targets for PZQ will be discovered. Indeed, several PZQ-interacting proteins in schistosomes have already been proposed including myosin light chain [62], actin [62,63] (but see [64]), voltage-operated Ca^{2+} channels [65], multidrug-resistant transporters [66], adenosine transporters [67], glutathione S-transferase (GST) [68], and several members of the tegumental allergen (TAL) family of proteins [69]. However, for the majority of these candidates, quantitative characterization of PZQ binding and the selectivity of the ligand binding site is lacking. Also, acknowledging the tight SAR of PZQ and the reciprocally tight SAR of the TRPM$_{PZQ}$ binding pocket, it is worth pointing out that many conjugated PZQ analogs utilized in prior target discovery strategies would poorly interact with TRPM$_{PZQ}$, if at all. Whether any of these reported interactions contribute to the therapeutic efficacy of PZQ remains the critical question, and this will require careful validation. Three fundamental criteria, outlined in the preceding sections for TRPM$_{PZQ}$, must be met. First, is there reasonable congruence between the affinity for PZQ at the proposed target versus PZQ efficacy against the worm? Second, is there a similar SAR for PZQ analogs at the proposed target versus the parasite? Third, is there a clear functional outcome consequent to PZQ engaging these targets that is consistent with the triad of phenotypic outcomes (depolarization, worm contraction, tegument damage)? For example, with Sm.TAL1, where careful efforts have been made to characterize PZQ binding, the resolved affinity is low (K$_d$ of Sm.TAL1 for PZQ = 140 μM [69]) compared with PZQ action on worms. For schistosome GST, where PZQ was cocrystallized with the enzyme, the binding site lies within an amphipathic groove at the dimer interface, which promiscuously accommodates many hydrophobic chemotypes [21] not reflecting the established SAR. Further, PZQ binding does not affect GST function [70]. Therefore, many of these proposed interactors may not stand up to scrutiny as a "primary" target.

Could there be another "primary" parasite target that matches the sensitivity, SAR, and functional impact of TRPM$_{PZQ}$? Another "primary" target cannot be excluded, while so many ion channels and GPCRs remain unprofiled in parasitic flatworms. However, one notes the insensitivity of *Fasciola* toward PZQ: If there is another "primary" target for PZQ, then the effects of engagement of this target should be manifest in *Fasciola* in the absence of any contribution from TRPM$_{PZQ}$. However, *Fasciola* spp. remain insensitive to very high (millimolar) concentrations of PZQ [26], consistent with a single "primary" target model. Notwithstanding this argument, the most likely place to look for additional PZQ targets might be other TRP (and likely TRPM) family members, as many of these channels have yet to be studied. This is a challenge in the absence of known activators to confirm successful heterologous expression of each ion channel candidate, as a negative result is not definitive in the absence of a positive control. However, a second TRPM family member has recently been deorphanized and shown to respond to the benzodiazepine, meclonazepam, an old anthelmintic [71]. This channel, named TRPM$_{MCLZ}$, did not respond to PZQ as predicted [24,71]. However, the fact that TRPM$_{MCLZ}$—a cousin of TRPM$_{PZQ}$—also mediates worm contraction, depolarization, and surface damage is broadly supportive of functional assignment of both anthelmintic targets to the same ion channel subfamily. This underscores the promise of TRPM channels for design and development of new anthelmintics.

What has it got in its pocketses?. It is fair to ask—what endogenous ligands activate TRPM$_{PZQ}$? TRPM$_{PZQ}$ is a large channel, with each monomer composed of approximately 2,200 amino acids (predicted as approximately 250 kDa). The expected tetramer would be of an exceptionally large size (>1 MDa). Within this quaternary structure, there are likely a multitude of nooks and crannies that could accommodate ligands, lipids, and accessory proteins. For example, as many as 16 unique "ligand" binding sites have been counted in human TRPV channels [72], many of which are conserved in other TRP family members [73,74]. Only the VSLD binding pocket has been mapped so far in TRPM$_{PZQ}$. Therefore, there is much work to do: This encompasses provision of a structural solution to TRPM$_{PZQ}$ architecture, identification of TRPM$_{PZQ}$ regulators, and then definition of their role in regulating TRPM$_{PZQ}$ responsiveness.

Already TRPM$_{PZQ}$ has emerged as a "polymodal" ion channel, defined as a channel regulated by both chemical as well as physical cues. Chulkov and colleagues demonstrated that membrane stretch activates *Sm*.TRPM$_{PZQ}$, a potentially relevant cue for an aquatic parasite that must traverse a pressurized vascular system [20]. Schistosome contraction is known to be regulated by stretch [75]. As there are a multitude of sensory demands across the parasitic life cycle, other environmental cues may emerge as TRPM$_{PZQ}$ regulators, potentially encompassing both parasite-derived and host-derived ligands [76]. Unbiased screening approaches, as well as biased probing of known vertebrate TRP channel regulators will be of value in identifying such ligands. Elaboration of the TRPM$_{PZQ}$ interactome to define interacting proteins will be another priority to identify other regulators of the channel complex. Unravelling the functional consequences of both ligand and accessory protein regulation of TRPM$_{PZQ}$ will surely reveal new targets for chemotherapeutic attack.

Conclusions

TRPM$_{PZQ}$ has recently emerged as a druggable parasite ion channel, with the evidence outlined in this review supportive of this ion channel acting as the relevant parasite target of PZQ. Recent work has elaborated the key properties of this ion channel and identified future experimental priorities. The prospect of identifying novel pharmacological tools for probing the function of TRPM$_{PZQ}$, as well as for other ion channels within the parasitic flatworm TRPM

subfamily [71] and the broader TRP channel superfamily [77], will further our understanding of the roles of these sensory ion channels throughout the parasitic lifecycle. This is currently a very exciting time for anthelmintic drug development, with new broad spectrum oxamniquine derivatives [78], highly potent antischistosomal chemotypes [79], as well as novel TRPM$_{PZQ}$ activators all recently emerging [32,49]. Hopefully, this will yield exciting advances for treating parasitic flatworm infections within the not-too-distant future.

Top Five Papers

1. Andrews P, Thomas H, Pohlke R, Seubert J. Praziquantel. Med Res Rev. 1983;3 (2):147–200. Epub 1983/04/01. doi: 10.1002/med.2610030204. PubMed PMID: 6408323.

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Key Learning Points

- Outline the key properties of the parasitic flatworm ion channel, TRPM$_{PZQ}$.

- Collate current evidence supporting TRPM$_{PZQ}$ as the target of the clinical drug, praziquantel.

- Suggest directions for future experimental work.

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