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The identification of inhibitors of *Schistosoma mansoni* miracidial transformation by incorporating a medium-throughput small-molecule screen

Andrew S. Taft^{*}, Francesca A. Norante, and Timothy P. Yoshino

Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin, Madison, WI 53706, United States

Abstract

In *Schistosoma mansoni*, the miracidium-to-primary sporocyst transformation process is associated with many physiological, morphological, transcriptional and biochemical changes. In the present study we use a medium-throughput small molecule screen to identify chemical compounds inhibiting or delaying the *in vitro* transformation of miracidia to the sporocyst stage. The Sigma-Aldrich Library of Pharmacologically Active Compounds (LOPAC) contains 1,280 well-characterized chemical compounds with various modes of action including enzyme inhibitors, antibiotics, cell-cycle regulators, apoptosis inducers and GPCR ligands. We identified 47 compounds that greatly reduce or delay this transformation process during a primary screen of live miracidia. The majority of compounds inhibiting larval transformation were from dopaminergic, serotonergic, ion channel and phosphorylation classes. Specifically, we found that dopamine D2-type antagonists, serotonin reuptake inhibitors, voltage-gated calcium channel antagonists and a PKC activator significantly reduced *in vitro* miracidial transformation rates. Many of the targets of these compounds regulate adenylyl cyclase activity, with the inhibition or activation of these targets resulting in increased cAMP levels in miracidia and concomitant blocking/delaying of larval transformation.

Keywords

Schistosoma mansoni; miracidia; sporocyst; transformation; drug screen; adenylyl cyclase; cAMP

1. Introduction

The transformation of the free-swimming miracidium to the parasitic mother sporocyst represents a major morphological remodeling event associated with many transcriptional changes (Taft et al., 2009; Vermeire et al., 2006; Fitzpatrick et al., 2009). During the *in vitro* transformation process miracidia cease swimming, “round up” and release their ciliated epidermal plates. Expansion of intercellular ridges formed from cytoplasmic extensions of submuscular cytons (perikarya) is responsible for the simultaneous shedding of epidermal

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Corresponding Author at: Department of Pathobiological Sciences, School of Veterinary Medicine, 2115 Observatory Drive, Madison, WI 53706-1087, United States. Tel.: +1 608 263 6003; fax +1 608 265 8122 tafta@svm.vetmed.wisc.edu (A.S. Taft).

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plates and creation of the tegumental syncytium of the fully formed sporocyst (Basch and DiConza, 1974; Samuelson and Caulfield, 1985). Although the morphological events of transformation are well-documented, little is known about the molecular mechanisms regulating this transition from the free-living to parasitic larval stages.

One of the major signals for initiating this early developmental process is a change in osmolarity. Freshly hatched miracidia will swim in artificial pond water (~10 mOsm/liter) for hours and even days until death, but after being placed into a suitable transformation media including simple phosphate-buffer saline or culture media (e.g., RPMI-1640, MEM or HBSS) with an osmolarity ≥ 80 mOsm/liter the parasites will slowly cease swimming and begin the transformation process within 2–4 hours (Voge and Seidel, 1972). At an osmolarity closer to *Biomphalaria glabrata* hemolymph levels (~110 mOsm/liter) most parasites will cease swimming within 10–20 min and begin shedding their ciliated plates (Voge and Seidel, 1972). The morphological changes observed *in vitro* appear to closely mimic the *in vivo* process based on ultrastructural observations of larval development (Pan, 1980) and the fact that *in vitro* cultured primary sporocysts can give rise to secondary sporocysts and cercariae *in vitro* (Ivanchenko et al., 1999) or when injected into snails, develop into mature, cercarial-producing infections (Kapp et al., 2003).

Chemical inhibitors have been used to study various developmental processes in early stages of *S. mansoni* including egg hatching and the miracidial transformation. Calcium channel blockers (diltiazem and verapamil), a calmodulin antagonist (W-7) and calcium chelators (EGTA, lanthanum chloride, ruthenium red) block the hatching of eggs (Katsumata et al., 1989; Katsumata et al., 1988), while the treatment of miracidia with the calcium antagonist TMB-8, calcium channel blocker nicardipine, or W-7 resulted in vesiculation and death of free-swimming miracidia (Kawamoto et al., 1989). In addition, forskolin (an adenylate cyclase activator) or trichostatin A (TSA) and valproic acid (VA), both histone deacetylase (HDAC) inhibitors, also block transformation (Azzi et al., 2009; Kawamoto et al., 1989). Recently, Ludtmann *et al.* (2009) showed that the inhibition of protein kinase C (PKC), resulted in more rapid miracidial transformation than controls.

Traditional large-scale methods of screening organisms to identify genes, proteins or pathways associated with specific developmental processes include site-specific mutagenesis, genomewide RNA interference (RNAi) screens and the use of high-throughput chemical compound screens. Genomewide RNAi screens have been used to identify pathways involved in RNAi, aging and polarity during development (Dudley et al., 2002; Labbe et al., 2006) in *Caenorhabditis elegans* and the Wnt-Wingless signaling pathway in *Drosophila melanogaster* (DasGupta et al., 2005). Although, RNAi approaches have been applied to various stages of *S. mansoni* including the sporocyst, schistosomula, egg and adult stages (reviews by Brindley and Pearce, 2007; Han et al., 2009; Krautz-Peterson et al., 2009; Yoshino et al., 2009), to date, there have been no reports of functional RNAi transcript knockdown in miracidia, due in large part to the difficulty of introducing dsRNA into eggs and/or the relatively short life-span of a miracidium once it has hatched. Moreover, there have not been any large scale RNAi screens performed in *S. mansoni*, due, in part, to lack of tools necessary to perform these screens, i.e. genomewide RNAi libraries, availability of abundant life stages for screening and a lack of clearly defined phenotypes. The recently completed *S. mansoni* genome (Berriman et al., 2009) should allow researchers eventually to design dsRNA constructs for the majority of the predicted genes, but other logistical barriers will need to be overcome. Traditional transgenic techniques including transposon based mutagenesis/transgenesis have been reported in *S. mansoni* and may provide researchers the ability to perform large scale insertional mutagenesis screens to identify important genes but these techniques are not desirable for large scale screening because of maintaining parasites in the complex life cycle (Kines et al., 2008; Mann et al.,

2008; Morales et al., 2007). Because of the previously mentioned shortcomings of performing large scale RNAi or mutagenesis screens, a medium throughput phenotypic chemical screen in *S. mansoni* is an efficient and inexpensive alternative to identify compounds or pathways involved in a biological or developmental phenomenon. To date, no chemical screens have been used to identify compounds that alter *S. mansoni* phenotypes in intramolluscan larval stages, although a recent “medium-throughput” drug screen has been used to identify phenotype-altering drugs affecting the mammalian host stages; namely the schistosomula and adult worm (Abdulla et al., 2009). High-throughput screening assays involving searches for chemical inhibitors of specific schistosome enzyme systems or activities also have been successfully performed (Sayed et al., 2008) leading to discovery of novel anthelmintics. However, these studies also were focused on drug-targets in the mammalian parasitic stages.

In the present paper, we report the screening of free-swimming *S. mansoni* miracidia with the Sigma LOPAC library to identify chemical inhibitors of the *in vitro* transformation process. We screened 1,280 catalogued compounds and identified 47 that dramatically alter the normal *in vitro* transformation process of cultured miracidia. A subset of the most active molecules included compounds with well-characterized cyclic adenosine monophosphate (cAMP)-elevating activities. Thus, the demonstration that compound-treated parasites also have increased levels of cAMP compared to transformation-competent control parasites, supports a regulatory role of cAMP in the induction of miracidial transformation.

2. Materials and Methods

2.1 Parasites

Schistosoma mansoni miracidia (NMRI strain) were obtained from the livers of 7–8 week infected Swiss-Webster mice as described by Yoshino and Laursen (1995). Miracidia were concentrated in 15ml conical tubes by placing on ice for 10 min and centrifuging at 4°C for 1 min 500 × g. This was followed by a single wash with cold, sterile artificial pond water (APW) (Nolan and Carriker, 1946) and again concentration on ice as before. Miracidia were quantified by mixing a known volume of miracidia suspension with an equal volume of 4% paraformaldehyde and counting larvae using an Olympus CK inverted light microscope. Parasites were then diluted to a final concentration of 150 miracidia/50 µl in either Chernin's balanced salt solution (CBSS; Chernin, 1963) supplemented with 1 mg/ml each of glucose and trehalose and penicillin G (0.06 mg/mL) and streptomycin sulfate (0.05 mg/ml), or complete BGE medium (cBge; Hansen, 1976) supplemented with 10% heat inactivated fetal bovine serum (FBS) and penicillin G (0.06 mg/mL) and streptomycin sulfate (0.05 mg/ml) just prior to drug testing.

2.2 Preliminary Library Screen and optimization of primary screen parameters

In order to optimize drug screening parameters an initial subset of 80 compounds from the Sigma LOPAC library were tested. Screening was performed in conjunction with the Keck-University of Wisconsin Comprehensive Cancer Center (UWCCC) Small Molecule Screening Facility (SMSF). LOPAC library concentrated stocks were maintained at –20°C in 384-well plates at a concentration of 1 mM compound dissolved in 100% DMSO. 48 µL of either CBSS or cBge were delivered to each well of a clear, flat-bottomed 96-well polystyrene plate using a Biomek FX robotic liquid handler (Beckman-Coulter Inc. Fullerton, CA), followed by addition of 2 µL of each compound or DMSO alone, serving as a carrier control, to the plate using the Biomek FX. Previously isolated and quantified miracidia were placed into a 55 mL sterilized solution basin (Labcor 730–005) and approximately 150 miracidia in 50 µL were aliquoted to each well of the 96-well plate using a 12-well multi-channel pipette to minimize variability in parasite numbers per well.

Miracidia were then observed using an Olympus CK inverted light microscope immediately and then every 8–12 hours for a total of 3 days. Morphological and behavioral changes differing from the control wells were noted. Compounds displaying altered phenotypes from this preliminary screen were purchased from Sigma-Aldrich (St Louis, MO) and an optimization screen was performed by placing freshly hatched miracidia into various concentrations of carrier DMSO (1, 2, 3 or 4%) and compound (1, 2, 3 and 4 μ M) in CBSS or cBge medium. Parasites were again evaluated for morphological and behavioral changes as before.

2.3 Primary Library Screen

The primary library screen was performed using a similar method as the preliminary screen although the number of test items was expanded to include the entire library of 1,280 compounds. Based on results of the preliminary screen, 48 μ L of cBGE medium supplemented with antibiotics was added to each well, followed by addition of 2 μ L of compound in DMSO or DMSO alone (control) to 96-well plates using the Biomek FX. Approximately 150 freshly-hatched miracidia in 50 μ L of cBGE were transferred to each well of the 96-well plate as previously described using a 12-well multichannel pipette. Parasites were maintained at 26°C under normoxic conditions and observed immediately and at 8–12 h intervals for a total of 3 days for morphological and behavioral changes differing from carrier control cultures. Due to the large number of miracidia required and the number of test compounds in the library, approximately a third of the library was screened on three separate weeks.

2.4 Secondary screen

Based on the results of the primary library screen, selected drugs producing clear alterations in the miracidial transformation process, either morphologically, behaviorally or temporally, were subjected to a secondary series of dose-response experiments to confirm initial observations and determine larval sensitivities to these compounds. All of the following compounds used in the secondary screen were purchased from Sigma-Aldrich (catalog numbers in parentheses); citalopram hydrobromide (C7861), phorbol 12-myristate 13-acetate (PMA) (P8139), triflupromazine hydrochloride (T2896), clomipramine hydrochloride (C7291), forskolin (F6886), 8-bromo-cAMP sodium (B7880), pCPT-cAMP (C3912), dibutyryl cAMP sodium salt (D0627), cAMP (A9501), H-89 (B1427) and 3-isobutyl-1-methylxanthine (IBMX: I7018). In these experiments 47 μ L of cBGE was added to the wells of 96-well flat/clear bottom plates, followed by addition of 3 μ L of compound and approximately 150 freshly hatched miracidia in 100 μ L cBGE medium, resulting in final compound concentrations of 200, 100, 50, 10, 2 and 0.5 μ M (2 mM, 200 μ M, 20 μ M or 2 μ M for IBMX) and a final DMSO concentration of 2%. Parasites were then incubated at 26°C under normoxic conditions for 18 h. Parasites were then visually scored as “transforming” if they were no longer swimming, assumed a “rounded” morphology and were in the process of shedding epidermal plates, or “transformed” in which the process has been completed (sporocyst tegument is fully-formed and no ciliated plates remain attached). Parasites were labeled dead if they were no longer motile, tegument/epidermal surface showed signs of degeneration and no flame-cell activity was evident. Following larval evaluation, parasites were fixed in 4% paraformaldehyde and the total numbers of parasites per well were quantified. Tests for each compound concentration were performed in triplicate on two separate occasions (two independent biological replicates) and scored using a blind experimental protocol incorporating number-coded plates. Screening of the PKA inhibitor, H-89, was performed as above except parasite phenotypes were determined after 4 h.

2.5 Immunocytochemistry

Freshly hatched miracidia were treated with 10 μ M of forskolin, citalopram, triflupromazine, IBMX or DMSO for 4 hours in cBGE, followed by collection and simultaneous fixation and permeabilization in 2% paraformaldehyde/1% Triton-X100 in snail phosphate buffered saline (sPBS: 8.41 mM Na_2HPO_4 /1.65 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ /45.34 mM NaCl, pH 7.4) at 4°C overnight with constant rotation (Barnstead Labquake rotator, Thermo Fisher Scientific, Waltham, MA). Specimens were then washed twice with sPBS and blocked overnight at 4 °C in 5% normal goat serum (Promega, Madison, WI) in sPBS. The samples were then incubated in 1:100 polyclonal rabbit anti-cAMP (AB306, Millipore) in blocking buffer overnight at 4°C, washed 5 $\times$ 10 min in sPBS and incubated overnight at 4°C in 4 μ g/ml of Alexa Fluor® 488 conjugated goat anti-rabbit secondary antibody (Invitrogen) in blocking buffer. Following 5 washes @ 10 min in sPBS, larvae were mounted on glass slides using Vectashield® mounting medium (Vector Laboratories, Burlingame, CA, USA) and sealed using nail polish to prevent dessication. All images were collected at equal exposure intervals using a Nikon Eclipse TE300 inverted microscope (Nikon Instruments, Melville, NY, USA) equipped with a Photometrics CoolSNAP ES2 digital camera system (Roper Scientific, Ottobrunn Germany). Average grey levels for individual parasites, indicating relative fluorescence, were calculated using Metamorph imaging software version 7.0 (Molecular Devices, Sunnyvale, CA) and statistically analyzed using the Mann-Whitney *U*-test with significance set at $P \leq 0.05$.

3. Results

3.1 Preliminary and Optimization Screen

A preliminary screen was performed to optimize the proper concentrations of DMSO, compound and *in vitro* transformation media type that would be used for the full screen with an emphasis on parasite longevity while maintaining easy identifiable phenotypes. Plate #1 quadrant 4 from the LOPAC library, containing 80 compounds, was chosen as the preliminary screen subset because it contained calcimycin, a Ca^{+2} ion channel antagonist, a compound class that previously had been shown to disrupt miracidial transformation (Kawamoto et al., 1989). During this preliminary screen, two other compounds also caused phenotype alterations differing from controls; chelerythrine and clomipramine HCl. Miracidia treated with chelerythrine died within 24 h while clomipramine HCl inhibited the majority of miracidia (~70%) from transforming within 48 h. Follow-up tests were then performed on calcimycin, chelerythrine and clomipramine HCl to further identify the optimal concentration of compound, carrier and transformation media type. The DMSO concentration for the primary screen was chosen to be 2% due to the lethality of higher concentrations within 12 h. Using 1% DMSO, we found that the death and delayed transformation phenotypes were not as pronounced as in 2% DMSO, possibly because the compounds were able to enter the parasite more quickly in the transformation competent media (cBge). Parasites were scored as viable if movement or flame cell activity was observed, and parasites in 2% DMSO/cBGE medium showed 95% viability after 3 days in culture at 26°C. Additionally, ~80% of miracidia transferred to 2% DMSO in cBge medium or cBge medium alone transform to sporocysts after 24 h of incubation. A final compound concentration of 2 μ M was chosen because phenotypes were clearly identifiable in miracidia treated with calcimycin, chelerythrine and clomipramine HCl. The final parameter tested was transformation media type to be employed in our screen. In this case, cBGE media was chosen as the primary screening medium because the transformation process begins later in cBGE than in CBSS, allowing for a better opportunity of compounds to exert its specific activity either before the transformation process begins or at very early stages of transformation. It also provided a longer window for visual evaluation and analyses to be completed.

3.2 Primary Screen

In total, we screened 1,280 compounds from the Sigma LOPAC library including many well characterized compounds in all the major drug target classes (Fig. 1). The primary screen resulted in the identification of 47 compounds negatively affecting the transformation process and 10 compounds resulting in parasite death within 48 h (Table 1). The two transformation-related phenotypes observed were termed “low transformation” (< ~30% of total miracidia transformed after 48 h) and “delayed transformation” (< ~50% of miracidia transformed after 24 h, but ~80% transformed by 48 h). More than 70% (33/47) of the compounds causing a delayed or low transformation phenotype were from the dopaminergic, serotonergic, phosphorylation, ion channel classes or “other” category (Fig 2). Larval treatment with 16 compounds resulting in the majority of the low-transformation phenotype included representatives of the selective serotonin reuptake inhibitors (SSRIs), dopamine D2-type antagonists and calcium homeostasis disruption drug subclasses (Table 1). Similarly, most of compounds causing a delayed transformation phenotype also were from these same three subclasses. Other compounds in the serotonergic class causing delayed transformation include serotonin ligands (N-omega-methyl-5-hydroxytryptamine oxalate salt and mesulergine hydrochloride) and a serotonin agonist (O-methylserotonin hydrochloride). Prominent among those drugs causing early larval death included common antihelminthics (niclosamide and praziquantel), an antibiotic (oligomycin) and common toxins (sanguinarine and iodoacetamide).

3.3 Secondary Screen

A secondary screen was performed to confirm the results of the primary screen and to assess concentration-dependence of compounds found to have major effects on miracidial transformation (Fig 3). Four compounds were used for the secondary screen representing the following subclasses; dopamine D2-type antagonists (triflupromazine), SSRIs (citalopram and clomipramine) and PKC activation (PMA). As shown in Fig. 3, after 18 h of culture in the 2% DMSO control medium, 80% ($\pm$ 3%) of miracidia had fully transformed in each of four biological replicate experiments. We found that all chemical compounds displayed a concentration dependent transformation curve in which 50% of parasites treated with the minimum drug concentrations (2 μ M triflupromazine, 10 μ M citalopram, 0.5 μ M clomipramine or 2 μ M IBMX) behaved and appeared structurally identical to freshly-hatched miracidia (Fig. 4). PMA treated parasites were viable and actively swimming after 48 h but displayed multiple structural abnormalities including partially detached plates and the posterior of some miracidia was compressed (Fig 4). At higher drug concentrations, we did observe lethality occurring at >50 μ M for clomipramine and PMA, >100 μ M for citalopram and >10 μ M for triflupromazine.

In addition, we also assessed the transformation of miracidia with various cAMP analogues (8-bromo-cAMP sodium, pCPT-cAMP, dibutyryl cAMP sodium salt), synthetic cAMP or caged UV-reactive cAMP; (Invitrogen) at different concentrations (10 μ M – 10 mM). However, none of the compounds significantly inhibited transformation levels after 18 h compared to controls (data not shown).

3.4 Immunocytochemistry of cAMP levels in treated parasites

The major compound classes found in the screen are linked to proteins involved in adenylyl cyclase regulation and because of this, a polyclonal cAMP antibody was used to assess the effects that certain pharmacological inhibitors of miracidial transformation had on cAMP levels and immunolocalization. Miracidia were treated with a representative of each of the major compound classes including citalopram (selective serotonin reuptake inhibitor), forskolin (adenylate cyclase activator), triflupromazine (dopamine D2-type inhibitor), IBMX (phosphodiesterase inhibitor), synthetic cAMP and DMSO (carrier only control) for 4

hours and then processed for immunocytochemistry. These specimens were then visualized using immunofluorescence imaging under identical conditions of lighting and exposure times to directly compare cAMP levels between the various treatments and controls. Miracidia treated with citalopram, forskolin, triflupromazine and IBMX demonstrated increased total fluorescence compared to DMSO controls indicating increased levels of cAMP in compound-treated miracidia after 4 hours of exposure (Fig. 5 and Fig. 6). By contrast, miracidia exhibited no significant differences in fluorescence intensity following treatment with synthetic cAMP when compared to DMSO only controls (data not shown). Quantification of total fluorescence intensities for individual miracidia demonstrates a significant difference ($P \leq 0.05$) in total cAMP content of citalopram, forskolin, triflupromazine and IBMX treated parasites compared to control larvae (Fig. 5 and 6).

3.5 Treatment with a PKA antagonist

To further identify the potential downstream target of cAMP, we treated miracidia with the PKA inhibitor H-89. The incubation of miracidia with H-89 (1 mM) resulted in an increase in the rate of transformation, as treated miracidia ceased swimming and began to shed their ciliated epidermal plates more quickly than the DMSO carrier controls. At 4 hours, ~80% of the miracidia treated with H-89 had ceased swimming and were actively transforming compared to ~50% actively transforming in the control group (Fig 7).

4. Discussion

Very little is known about the signaling mechanisms involved in the process by which *S. mansoni* miracidia transform to primary sporocysts upon entry into the snail intermediate host. Previously it has been shown that an increase in osmolarity to > 80 mOsm/L serves as a transformation trigger *in vitro* (Voge and Seidel, 1972), and the use of forskolin, an adenylyl cyclase (ADCY) activator, inhibits this process (Kawamoto et al., 1989). In the present study we identified 47 chemical compounds that delay or inhibit transformation of the miracidium to the sporocyst stage. The biological targets for the majority of transformation-disrupting compounds are in the dopaminergic, serotonergic, phosphorylation or ion channel classes. Our overall findings indicate many of the identified compounds that negatively impact larval transformation serve as direct or indirect activators of adenylyl cyclase activity and suggest that prevention or delay of transformation may be mediated by elevated levels of cAMP, a notion that is supported by our immunocytochemical observations.

One of the major compound classes inhibiting or delaying transformation is the dopaminergic class, comprising antagonists and agonists of D1- and D2-type receptors, and in the present study, approximately 25% of our total hits were represented by this target class. Dopamine receptors are Type 1 G-protein-coupled receptors (GPCRs) in the rhodopsin family characterized by a seven transmembrane domain, an extracellular amino terminus and an intracellular carboxy-tail (Blenau and Baumann, 2001). Mammals and invertebrates have five dopamine receptors and they are subdivided into 2 classes, D1-type and D2-type, based on amino acid sequence homology and/or pharmacological profiles. Typically, D1-type dopamine receptors (D1 and D5) are coupled to Gs (G-stimulatory) proteins and their induction leads to an activation of ADCY, triggering an increase in cAMP production through the conversion of adenosine triphosphate (ATP) to cAMP (Blenau and Baumann, 2001). In contrast, D2-type dopamine receptors (D2, D3 and D4) are coupled to Gi/o (G-inhibitory) proteins that lead to the inhibition of ADCY and reduced levels of cAMP. In the present study, only well-known mammalian antagonists of D2-type receptors (e.g. triflupromazine, chlorpromazine, raclopride and perphenazine), but not D1-type receptors were found to inhibit miracidial transformation. This implies that the transformation process is blocked because the inhibition of a negative regulator of ADCY

(e.g., D2-type dopamine receptors) leads to the activation of adenylyl cyclase, thereby increasing cAMP. Although the LOPAC library contains many D1-type agonists (e.g., dihydrexidene, fenoldopam and A-77636), which would presumably also raise cAMP levels, no transformation inhibitory effect of these compounds was observed during our primary screen. One possible explanation for larval nonresponsiveness may be a lack of D1-type receptors in the miracidial stage or associated with specific larval tissues/cells involved in the transformation process. Supporting a classical mammalian D2-type pharmacologic profile, we showed that treatment of miracidia with the D2-type antagonist triflupromazine increases immunoreactive cAMP levels and further suggests a classic D2-type receptor in miracidia functioning as a G-inhibitory receptor. Pharmacological blocking the normal G-inhibitory function of the receptor predictably led to elevated cAMP and inhibition of the transformation process. A D2-type receptor from *S. mansoni* (*SmD2*) recently was cloned and characterized (Taman and Ribeiro, 2009) and, although this receptor's amino acid sequence is similar to other D2-type receptors, when expressed in mammalian HEK cells, its activation with dopamine led to an ADCY stimulatory response and an increase in cAMP, unlike other well-characterized D2-type receptors in *C. elegans*, *A. mellifera* and *D. melanogaster* (Beggs et al., 2005; Hearn et al., 2002; Suo et al., 2003). Taman and Ribeiro (2009) acknowledge that the cloned receptor has an unusual pharmacological profile when expressed in the heterologous system because apomorphine, a potent mammalian D2-type antagonist, functions as an agonist for the *SmD2* receptor, while other classic mammalian antagonists had no effect. Our finding that treatment of miracidia with classical mammalian D2-type antagonists inhibits transformation and the larval incubation in the D2-antagonist triflupromazine increases cAMP levels suggests that either the cloned *SmD2* functions differently in the heterologous expression system than *in vivo* or that there may be an additional classically-acting mammalian D2-type receptor(s) expressed in *S. mansoni* miracidia.

Another highly represented class of molecules delaying or inhibiting miracidial transformation is the serotonergic class, specifically selective serotonin reuptake inhibitors (SSRIs), serotonin ligands and a serotonin receptor agonist. Serotonin (5-HT) is a ubiquitous monoamine neurotransmitter found in plants and animals that is synthesized from L-tryptophan through its conversion to 5-hydroxy-L-tryptophan by tryptophan hydroxylase (TPH) and then to 5-HT using aromatic L-amino acid decarboxylase (AAAD) (Hamdan and Ribeiro, 1999). Serotonin is typically located in small vesicles in the presynaptic neuron and electrical impulses cause the vesicles to fuse with the neuronal membrane followed by release into the synaptic space. Citalopram and clomipramine are well-characterized SSRIs and in the present study inhibited transformation in ~80% of miracidia at concentrations of 50 μ M and 10 μ M, respectively. SSRIs function by blocking the transport (reuptake) of serotonin back into the presynaptic cell, thereby increasing extracellular serotonin and activating the serotonin receptor. Similar to dopamine D1-type receptors, certain mammalian serotonin receptors including 5-HT₄, 5-HT₆ and 5-HT₇ are stimulatory GPCRs with respect to ADCY (Yocca and Maayani, 1990). Thus the result of the influx of serotonin into the extracellular space is an increase in cellular cAMP, as we demonstrated by cAMP immunolocalization in citalopram-treated miracidia, and an associated blocking of transformation. The additional finding that a serotonin receptor agonist and a serotonin ligand also block transformation is consistent with this hypothesis in that both of these compounds activate the serotonin receptor leading to the activation of ADCY. Previous studies in *S. mansoni* have demonstrated that citalopram and clomipramine function as selective serotonin reuptake inhibitors by inhibiting the *S. mansoni* 5-HT serotonin transporter *SmSERT* in a heterologous expression system (Patocka and Ribeiro, 2007; Fontana et al., 2009). Although Boyle et al. (2003) did not detect serotonin transporter activity in miracidia they were only examining the uptake of exogenous 5-HT and would have likely missed endogenous 5-HT transport. Consistent with our finding that the

treatment of miracidia with citalopram results in increased cAMP, serotonin receptors in *S. mansoni* have been shown to function as G-stimulatory proteins in the cercariae, schistosomula and adult stages (Kasschau and Mansour 1982a; Kasschau and Mansour 1982b).

To further address the hypothesis that cAMP is involved in the miracidial transformation process, we incubated miracidia with the cyclic nucleotide phosphodiesterase inhibitor, IBMX, and found that this compound significantly blocks transformation in miracidia. Cyclic nucleotide phosphodiesterases belong to a group of enzymes that degrade the second messengers cAMP to AMP and cGMP to GMP by breaking the phosphodiester bond in these molecules. The breakdown of these second messengers is an important process in regulating the spatial and temporal signaling of certain cyclic nucleotide pathways. Although IBMX is a nonspecific cyclic nucleotide inhibitor, we demonstrate using immunofluorescence that it acts as a cAMP phosphodiesterase inhibitor in *S. mansoni* miracidia due to the fact that treatment with IBMX raises endogenous levels of cAMP. Additionally, the phenotype elicited by treatment with IBMX mimics that of treatment with the adenylyl cyclase activator, forskolin, selected D2-type receptor antagonists and SSRIs suggesting a strong link between elevated cAMP levels and developmental arrest in miracidia.

Previously it has been reported that the inhibition of protein kinase C (PKC) using various pharmacological agents increases the rate of miracidial transformation compared to controls (Ludtmann et al., 2009). In our study, we found that the presumed activation of PKC by the diacylglycerol analogue, PMA, inhibits transformation of miracidia, although the phenotype observed using PMA is quite different than the phenotype associated with forskolin, D2-type antagonist or SSRI treatments. PMA-treated parasites typically assume a more rounded shape and swim slowly whereas the phenotype observed using cAMP-increasing compounds is more similar to freshly hatched miracidia. This suggests that PKC and cAMP signaling pathways, although both associated with the transformation process, may be acting through distinct regulatory mechanisms.

In the current screen we also identified L-type and T-type calcium channel blockers/antagonists that interfere with the transformation of miracidia. Similar to treatment with PMA, L- and T-type calcium antagonists cause miracidia to exhibit a rounded and slow swimming phenotype and these parasites either vesiculate and die, presumably due to disruption in calcium homeostasis, or eventually transform, possibly because of certain pharmacokinetic properties of the drug (e.g., excretion from the body or metabolism of the compound). This rounded slow-swimming phenotype was previously reported by Kawamoto et al. (1989) when they demonstrated that treatment with TMB-8 (an intracellular calcium antagonist), nicardipine (an L-type calcium channel blocker), and A23187 (a calcium ionophore) cause vesiculation and death of miracidia in 3 hours further supporting the importance of maintaining Ca²⁺ balance during early larval development.

The specific mechanism downstream of cAMP causing the inhibition of the transformation process is presently unknown although the involvement of a CREB (cAMP responsive element binding) transcription factor(s) would be one likely candidate. Cyclic AMP has been shown to induce gene transcription through the activation of cAMP-dependent protein kinase (PKA), which subsequently phosphorylates CREB. Treatment with the PKA selective inhibitor H-89 resulted in a larger percentage of miracidia undergoing transformation, implying that PKA may be the primary target for cAMP during this process. In this case, by blocking PKA activation by cAMP, miracidia are induced to begin the transformation process sooner. However, contrary to this prediction, we found that synthetic cAMP or the cAMP analogues 8-bromo-cAMP sodium, pCPT-cAMP, dibutyryl cAMP sodium salt and

UV-released cAMP do not inhibit the transformation process. It is unknown whether these analogs function properly in *S. mansoni* or whether they are able to access the proper tissues to disrupt the transformation process, but immunocytochemistry results indicate that cAMP levels are not significantly different in parasites treated with synthetic cAMP compared to control parasites. We hypothesize that this may be due to the rapid breakdown of endogenously added synthetic cAMP by phosphodiesterases or that synthetic cAMP may not permeate the external surface of the parasite.

CREB functions by binding to the promoter of cAMP responsive target genes which then forms a complex with CREB binding proteins (CBP) and activates or represses transcription. To date, CREB has not been cloned or characterized from *S. mansoni*, but two CREB binding proteins, SmCBP1 and SmCBP2 have been identified from *S. mansoni* (Bertin et al., 2006). SmCBP1 and SmCBP2 are highly expressed transcripts in miracidia and their transcript levels decrease rapidly in 24-h *in vitro* transformed primary sporocysts (Bertin et al., 2006) and remain depressed in 6- and 20-day *in vitro* cultured sporocysts (Taft et al., 2009). We hypothesize that increased levels of cAMP cause constitutive activation of CREB controlled “miracidial specific” genes, thus preventing the parasite from transforming. Although the commonly used CREB inhibitor curcumin is lethal to *S. mansoni* adult worms *in vitro* (Magalhaes et al., 2009), it has been shown to inhibit multiple transcription factors, in addition to CREB, thereby complicating the analysis of curcumin-treated organisms (Shishodia et al., 2007).

In summary, as a result of evaluating over 1,200 small-molecules in a medium-throughput screen, we have identified a subset of compounds that inhibited *in vitro* miracidial transformation. Novel transformation inhibitors include dopamine D2-type receptor antagonists and serotonin reuptake inhibitors wherein treatment with these compounds resulted in increased levels of cytosolic cAMP in the miracidia. Increased levels of cAMP appear to be signaling through a PKA as evidenced by a more rapid transformation of miracidia in the presence of PKA inhibitors compared to controls. We hypothesize that increased levels of cAMP may be signaling through the CREB pathway to turn miracidial-specific genes “on”, and with the maintenance of high cAMP levels by exposure to certain compounds, “miracidial” genes are not turned “off” to begin the transformation process. The upstream cue for this transformation is an increase in osmolarity, but the specific type of receptor involved in initiating the change in cAMP levels and consequent miracidial transformation is not known. The involvement of dopamine D2-type receptors and serotonin reuptake inhibitors suggests that the neural network radiating from the neural mass may be involved in this complex process.

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Fig. 1.
Distribution of 1,280 catalogued compounds classes present in the Sigma LOPAC library. Miracidia of *Schistosoma mansoni* were screened with this library to gain insights into possible regulatory mechanisms involved in early larval development.

Fig. 2.
Results of the primary library screen of compounds causing delayed or low transformation phenotypes (see text for description) in *Schistosoma mansoni* miracidia sorted by compound class (compounds causing death excluded).

Fig. 3.
Graphs showing the transformation dose-responses of *Schistosoma mansoni* miracidia at 18 h post-treated with (A) citalopram (B) clomipramine (C) triflupromazine (D) PMA (E) IBMX (12 h).

Fig. 4. Photomicrographs of live *Schistosoma mansoni* miracidia after 4-h treatment with 10 μ M (a) citalopram (b) clomipramine (c) triflupromazine (d) forskolin (e) PMA, (f) IBMX or (g) DMSO only (control). Note abnormal appearances in PMA treated parasites and the rounded appearance of actively transforming miracidia in DMSO only treated parasites (arrows indicate ciliated plates beginning to separate).

Fig. 5.
 (a) Immunocytochemical localization of anti-cAMP reactivity in miracidia of *Schistosoma mansoni* following a 4 h treatment with 10 μ M of (from upper-left clockwise) DMSO, forskolin, citalopram or triflupromazine in cBGE medium. Note increased fluorescence indicating higher levels of cAMP compared to the DMSO control. (b) Relative fluorescence levels of compound treated miracidia. All fluorescence levels are significantly different from controls ($p < .05$).

Fig 6. Immunocytochemistry analysis of miracidia following a 4 h treatment with 20 μ M of IBMX or DMSO and the corresponding relative fluorescence levels demonstrating a significant difference in median relative fluorescence levels ($p < .01$).

Fig 7.
Proportion of actively transforming *Schistosoma mansoni* miracidia after 4 h incubation in the PKA inhibitor H-89 (1 mM) or DMSO control in cBGE media.

Summary of observed *Schistosoma mansoni* miracidial phenotypes resulting from *in vitro* exposure to compounds during the primary screen of 1,280 molecules from the Sigma-LOPAC library.

Table 1

SIGMA Catalog Number	Phenotype	Compound	Class	Enzyme Action	Selectivity
D108	low transformation	1,3-Dipropyl-7-methylxanthine	Adenosine	Antagonist	A2
A8404	low transformation	Amitriptyline hydrochloride	Adrenoceptor	Inhibitor	Uptake
D4526	low transformation	Doxepin hydrochloride	Adrenoceptor	Inhibitor	Uptake
L8397	low transformation	L-Leucinethiol, oxidized dihydrochloride	Biochemistry	Enzyme Inhibitor	Aminopeptidase
S7882	low transformation	(-)-Scopolamine, n-Butyl-, bromide	Cholinergic	Antagonist	Muscarinic
F6886	low transformation	Forskolin	Cyclic Nucleotides	Enzyme Activator	Adenylate cyclase
L135	low transformation	L-741,626	Dopamine	Antagonist	D2
C8138	low transformation	Chlorpromazine hydrochloride	Dopamine	Antagonist	D2
T2896	low transformation	Triflupromazine hydrochloride	Dopamine	Antagonist	D2
T 8516	low transformation	Trifluoperazine dihydrochloride	Dopamine	Antagonist	D2
H 9003	low transformation	Hydroquinone	Leukotriene	Enzyme Inhibitor	Arachidonate 12-Lipoxygenase
P8139	low transformation	Phorbol 12-myristate 13-acetate	Phosphorylation	Enzyme Activator	PKC
M6628	low transformation	O-Methylserotonin hydrochloride	Serotonin	Agonist	
I7379	low transformation	Imipramine hydrochloride	Serotonin	Blocker	Reuptake
F1678	low transformation	R-(-)-Fluoxetine hydrochloride	Serotonin	Inhibitor	Reuptake
C7861	low transformation	Citalopram hydrobromide	Serotonin	Inhibitor	Reuptake
C7291	low transformation	Clomipramine hydrochloride	Serotonin	Inhibitor	Reuptake
F7927	delayed transformation	FSCPX	Adenosine	Antagonist	A1
N7261	delayed transformation	Nortriptyline hydrochloride	Adrenoceptor	Inhibitor	Uptake
F9677	delayed transformation	Felodipine	Ca2+ Channel	Blocker	L-type
M5441	delayed transformation	Mibefradil dihydrochloride	Ca2+ Channel	Blocker	T-type

SIGMA Catalog Number	Phenotype	Compound	Class	Enzyme Action	Selectivity
M5644	delayed transformation	(±)-Methoxyverapamil hydrochloride	Ca ²⁺ Channel	Antagonist	L-type
R121	delayed transformation	S(+)-Raclopride L-tartrate	Dopamine	Antagonist	D2
C1671	delayed transformation	Chlorprothixene hydrochloride	Dopamine	Antagonist	D2
T 9025	delayed transformation	Thionidazine hydrochloride	Dopamine	Antagonist	D2
P6402	delayed transformation	Perphenazine	Dopamine	Antagonist	D2
F114	delayed transformation	cis-(Z)-Flupenthixol dihydrochloride	Dopamine	Antagonist	D2
O111	delayed transformation	(±)-Octoclohepin maleate	Dopamine	Antagonist	D2
S8010	delayed transformation	(±)-Sulpiride	Dopamine	Antagonist	D2
S7771	delayed transformation	(-)-Sulpiride	Dopamine	Antagonist	D2
T101	delayed transformation	THIP hydrochloride	GABA	Agonist	GABA-A
C121	delayed transformation	7-Chlorokynurenic acid	Glutamate	Antagonist	NMDA
I160	delayed transformation	3-(1H-Imidazol-4-yl)propyl di(p-fluorophenyl)methyl ether hydrochl	Histamine	Antagonist	H3
D9175	delayed transformation	Dantrolene sodium	Intracellular Calcium	Inhibitor	Release
C7522	delayed transformation	Calcimycin	Intracellular Calcium		Ca ²⁺
S0758	delayed transformation	Sulfaphenazole	Multi-Drug Resistance	Enzyme Inhibitor	Cytochrome P4502C
L8539	delayed transformation	L-687,384 hydrochloride	Opioid	Agonist	signal
P4484	delayed transformation	Purvalanol A	Phosphorylation	Enzyme Inhibitor	CDK
C1172	delayed transformation	ML-9	Phosphorylation	Enzyme Inhibitor	MLCK
Q109	delayed transformation	Quipazine, 6-nitro-, maleate	Serotonin	Inhibitor	Reuptake

SIGMA Catalog Number	Phenotype	Compound	Class	Enzyme Action	Selectivity
G5793	delayed transformation	GR 127935 hydrochloride	Serotonin	Antagonist	5-HT1B/1D
F2802	delayed transformation	Fluvoxamine maleate	Serotonin	Inhibitor	Reuptake
I119	delayed transformation	Indatraline hydrochloride	Serotonin	Inhibitor	Reuptake
M153	delayed transformation	Mesulergine hydrochloride	Serotonin	Ligand	
M1514	delayed transformation	N-omega-Methyl-5-hydroxytryptamine oxalate salt	Serotonin	Ligand	
L137	delayed transformation	L-733,060 hydrochloride	Tachykinin	Antagonist	NK1
S3442	delayed transformation	SB 216763	Phosphorylation	Enzyme Inhibitor	GSK-3
O5001	death	Oligomycin A	Antibiotic	Enzyme Inhibitor	Mitochondrial FOF1-ATP synthase
N3510	death	Niclosamide	Antibiotic		Protonophore
P4668	death	Praziquantel	Antibiotic		Ca ²⁺ Ionophore
I1149	death	Iodoacetamide	Biochemistry	Enzyme Inhibitor	
N7906	death	2-(alpha-Naphthoyl)ethyltrimethylammonium iodide	Cholinergic	Enzyme Inhibitor	Choline Acetyltransferase
C2538	death	Carboplatin	DNA	Intercalator	
M 6545	death	Mitoxantrone	DNA Metabolism	Inhibitor	
S5890	death	Sanguinarine chloride	Ion Pump	Inhibitor	Na ⁺ /K ⁺ ATPase
S6879	death	Sphingosine	Phosphorylation	Enzyme Inhibitor	PKC
C2932	death	Chelerythrine chloride	Phosphorylation	Enzyme Inhibitor	PKC