Invited article

Pharmacological profiling an abundantly expressed schistosome serotonergic GPCR identifies nuciferine as a potent antagonist

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ABSTRACT

5-hydroxytryptamine (5-HT) is a key regulator of muscle contraction in parasitic flatworms. In *Schistosoma mansoni*, the myoexcitatory action of 5-HT is effected through activation of a serotonergic GPCR (Sm.5HTR$_L$), prioritizing pharmacological characterization of this target for anthelmintic drug discovery. Here, we have examined the effects of several aporphine alkaloids on the signaling activity of a heterologously expressed Sm.5HTR$_L$ construct using a cAMP biosensor assay. Four structurally related natural products — nuciferine, D-glaucine, boldine and bulbocapnine — were demonstrated to block Sm.5HTR$_L$ evoked cAMP generation with the potency of GPCR blockade correlating well with the ability of each drug to inhibit contractility of schistosomule larvae. Nuciferine was also effective at inhibiting both basal and 5-HT evoked motility of adult schistosomes. These data advance our understanding of structure-affinity relationships at Sm.5HTR$_L$, and demonstrate the effectiveness of Sm.5HTR$_L$ antagonists as hypomotility-evoking drugs across different parasite life cycle stages.

1. Introduction

Over 200 million people worldwide are infected with parasitic schistosome flatworms, which cause the disease schistosomiasis. The drug praziquantel (PZQ) has served as the stalwart of anti-schistosomal therapy since the 1980s. However several features of PZQ are less than ideal, and the lack of research into next generation antischistosomal therapies makes the specter of emergence of PZQ-resistant strains potentially devastating.

Screening for antischistosomal drugs that impair parasite mobility is a logical approach toward anthelmintic drug discovery (Pax et al., 1996; Ribeiro et al., 2005): indeed, neuromuscular impairment is considered a key component of the clinical efficacy of PZQ. Defects and shape malformations therefore form the basis of many antischistosomal phenotypic screens (Abdulla et al., 2009; Paveley et al., 2012; Mansour et al., 2016; Singh et al., 2016). From a target-based screening approach there is also considerable interest in dissecting the neuromuscular physiology of schistosomes to identify key signaling nodes that coordinate excitable cell activity. Resolution of the contribution from G protein coupled receptors (GPCRs) to parasite neuromuscular signaling is especially alluring, given the preponderance of GPCR ligands among existing therapeutics.

In this context, several recent studies have begun to establish the role(s) of individual GPCRs within the excitable cell niche (El-Shehabi et al., 2012; Patocka et al., 2014; Chan et al., 2015; MacDonald et al., 2015). Of relevance to this study, is the identification of a serotonergic GPCR (Sm.5HTR$_L$) that has been shown by RNAi approaches to control larval and adult worm motility (Patocka et al., 2014). Knockdown of this receptor, expressed across the parasite life cycle (Protasio et al., 2012), caused hypoactive motor phenotypes in schistosomules and adult worms consistent with the myoexcitatory role of 5-HT (Patocka et al., 2014). Knockdown of the Sm.5HTR$_L$ homologue in free living planarians (*Dj*-S7.1R, see companion paper, Chan et al., 2016a) also causes hypomobility (Chan et al., 2015).

We recently applied a cAMP biosensor technology to profile the ligand binding specificities of this GPCR. This approach revealed properties and a pharmacological divergence of Sm.5HTR$_L$ from the closest human GPCR homolog (Hs.5HTR$_7$) that further

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enhanced the appeal of Sm.5HTR$_L$ as a chemotherapeutically vulnerable node for anthelmintic development (Chan et al., 2016b). The chemical screening data demonstrated a potent, and long lasting inhibition evoked by the ergot alkaloid bromocriptine at Sm.5HTR$_L$ (evidenced also for the planarian S7.1R in the companion paper (Chan et al., 2016a)), and highlighted several Sm.5HTR$_L$ antagonists with dimethoxyisoquinoline substructures. Here, we apply the cAMP biosensor to expand upon this growing understanding of structure-activity relationships at Sm.5HTR$_L$ by examining the effects of several aporphine alkaloids containing methoxyisoquinoline moieties.

Aporphines are tetracyclic alkaloids that display a broad range of pharmacological activities encompassing affinity for bio-aminergic receptors. A clinically well-known example is apomorphine, a synthetic morphine derivative used clinically as an anti-Parkinsonian agent due to efficacy at dopaminergic GPCRs. Several aporphine compounds have previously been demonstrated to impair neuromuscular function in a variety of helminth parasites (Ayers et al., 2007; Chan et al., 2014; Lin et al., 2014), although the molecular target of these drugs in parasites is uncharacterized. The affinity of aporphinoids for mammalian 5-HT GPCRs (Munusamy et al., 2013; Ponnala et al., 2014, 2015; Farrell et al., 2016) was especially intriguing to us in this context. Here, we examine the action of four aporphinoid natural products at Sm.5HTR$_L$ and demonstrate a close correlation between receptor blockade and a hypomotive action on schistosome larvae and adults.

2. Materials and methods

2.1. Chemicals

Aporphine derivatives were obtained from the vendors indicated: R-(−)-apomorphine (Sigma Aldrich, A4393), (−)nuciferine (Cerilliant, PHY83282), D-Glaucine (Santa Cruz Biotechnology, sc-490895), (+)boldine (Sigma Aldrich, 67592), (+)-Bulbocapnine (Santa Cruz Biotechnology, sc-257199). Other chemicals sourced for assays were 3-Isobutyl-1-methylxanthine (Sigma Aldrich, I5879), serotonin (Sigma Aldrich, H9523) and forskolin (Cell Signaling Technology, 3828).

2.2. Cell culture and cAMP assays

HEK293 cells (ATCC CRL-1573.3) were cultured in growth media (DMEM, 10% heat inactivated fetal bovine serum, penicillin (100units/mL), streptomycin (100 μg/mL) and L-glutamine (290 μg/mL)) and used between passages 5—25. For GPCR heterologous expression and functional assays, cells were transfected (Lipofectamine, 2000) at 80% confluency approximately 16 h after seeding on T-25 culture flasks with a 1:1 ratio of Sm.5HTR$_L$ and the pGloSensor 22-F plasmid (Promega). Sm.5HTR$_L$ was codon optimized for human expression and subcloned into the pcDNA3.1(−) mammalian expression vector. Sm.5HTR$_L$ (GenBank accession, KX150867) is a longer form of the Sm.5HTR originally reported by Patocka et al. (Patocka et al., 2014). The following day, cells were trypsinized, centrifuged (300 g/5 min), resuspended in DMEM supplemented with 1% dialyzed FBS (Gibco) and plated in 96 well, solid white plates (Corning, cat # 3917). After overnight culture to allow adherence, media was exchanged for assay buffer (HBSS supplemented with 0.1% BSA, 20 mM HEPES (pH 7.4), and Glo-Sensor reagent (Promega)). cAMP-luminescence assays were performed in the presence of phosphodiesterase inhibitor (IBMX, 200 μM) using a GloMax®-Multi Detection System plate reader (Promega).

2.3. Schistosomule mobility assays

Biomphalaria glabrata snails exposed to *Schistosoma mansoni* miracidia (Strain PR-1) were provided by BEI Resources (NR-21961). Schistosomules were obtained by mechanical transformation from shed cercariae as described in (Chan et al., 2016b). Briefly, cercariae were shed by exposure to light (1.5 h), vortexed in 50 mL conical tubes (3 × 45secs, each separated by 3 min on ice), and tails were removed by gradient centrifugation (24 ml Percoll, 4 ml 10X EMEM, 1.5 ml penicillin-streptomycin, 1 ml of 1M HEPES in 0.85% NaCl, 9.5 ml distilled water) at 500 g/15 min at 4 °C. Tails were discarded (supernatant fraction) and pelleted schistosomules were resuspended in modified Basch media (lacking serotonin) and incubated (37 °C, 5% CO_2) before conducting mobility assays. Drugs were added to schistosomules in 24 well plates (~200 schistosomules/0.5 mL media per well) at a 20x concentration (i.e. 25 mL stock/500 mL schistosomules), mixed, and schistosomules were incubated for 30 min (37 °C/5% CO_2) prior to acquiring videos of schistosome movement (1 min video recording per well) using a Nikon Coolpix 5700 camera affixed to a Nikon Eclipse TS100 microscope (10x objective). Worm mobility was quantified by processing videos through ImageJ using the WrmTrck plugin to obtain a measurement for the body length of each schistosomule over the duration of the recording. Mobility was defined by quantifying the number of times per minute that the worm body length deviated from the average by over 20%. *S. mansoni* protocols were approved by the Iowa State University Institutional Biosafety Committee.

2.4. Adult schistosome mobility assays

Female Swiss Webster mice infected with *Schistosoma mansoni* cerceria (Strain PR-1) were obtained from BEI Resources (Cat. number NR-34792) and sacrificed 6—8 weeks post-infection. Adult *S. mansoni* were recovered from the mesenteric vasculature by portal perfusion (Chan et al., 2016b). Mice were anesthetized in a CO_2 chamber, sacrificed by cervical dislocation, and perfused with sodium citrate (25 mM). Adult schistosomes harvested from the mesenteric veins were washed in RPMI media supplemented with penicillin (100units/mL), streptomycin (100 μg/mL) and 25 mM HEPES and then transferred to RPMI media supplemented with 2 mM glutamine and 5% heat inactivated FBS. Worms were incubated overnight at 37 °C, 5% CO_2 before conducting mobility assays. Recordings of adult schistosome movement were acquired using a Zeiss Discovery v20 stereomicroscope and a QiCAM 12-bit cooled color CCD camera at a rate of four frames per second over one minute. Videos of female worms were acquired at 7.6x magnification, 30 mm field of view and videos of male worms were acquired at a 5.1x magnification, 45 mm field of view. Movement was quantified from video recordings according to the protocol described in Patocka et al. (2014). Image (.tiff) stacks were imported into ImageJ and converting to binary format, representing the worm body area as a measurement of pixels in each frame. The difference in pixels resulting from subtracting the value of one frame (n) from the next in the sequence (n+1), expressed as a percentage of the pixels in the initial frame (n), provided a measurement of worm movement over a period of 0.25secs. By performing this measurement for each frame in the video, an average length was determined for the cohort recorded. Values represent the mean(±)standard deviation of at least three independent experiments. Significance values were obtained by unpaired t-tests and represented as (*) $p < 0.05$, (**) $p < 0.01$. Animal work was carried out with the oversight and approval of the Laboratory Animal Resources facility at the Iowa State University College of Veterinary Medicine.
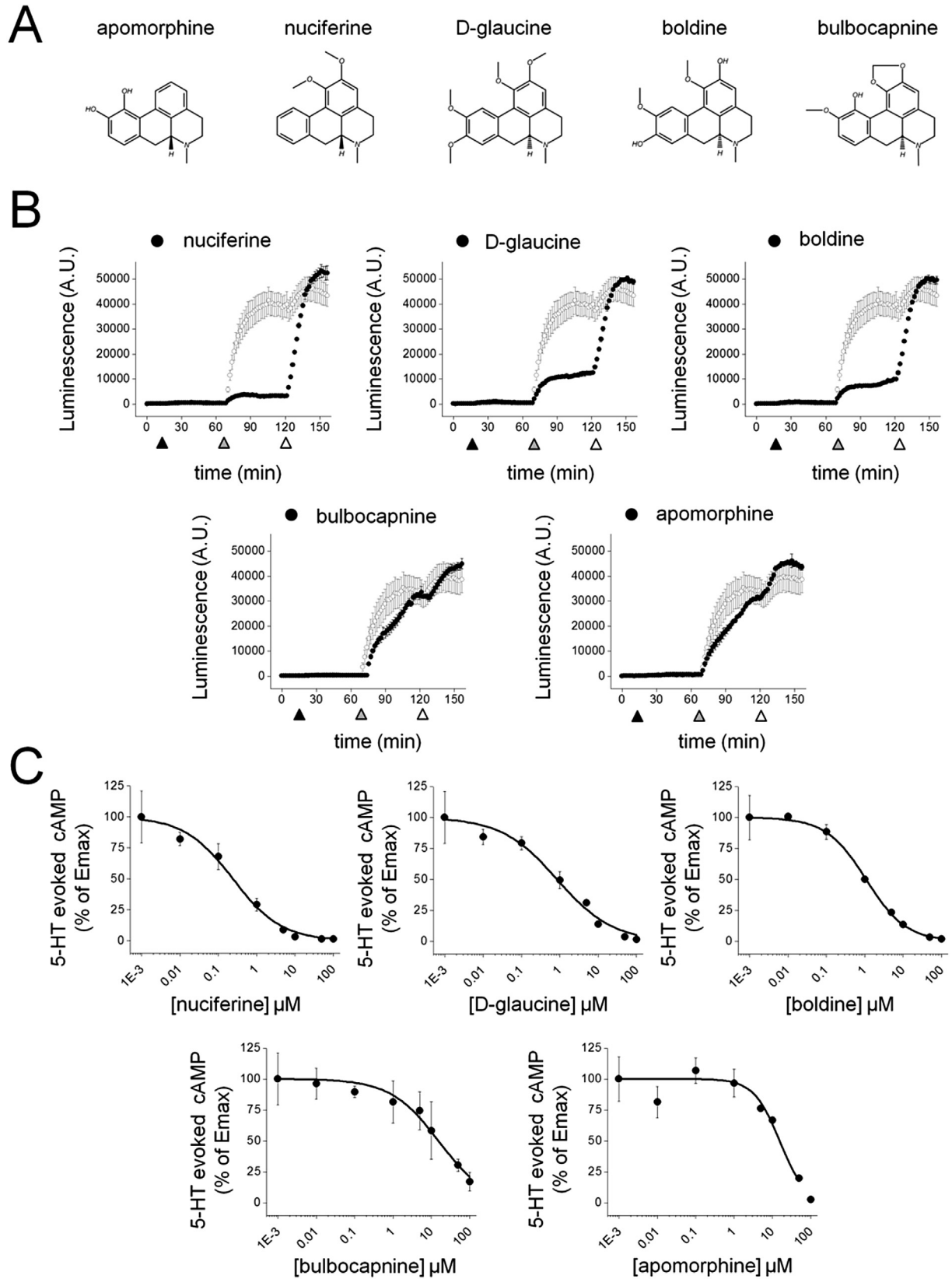
Fig. 1. Aporphine alkaloid natural products are Sm.5HTR$_L$ antagonists. (A) Structures of apomorphine, a semi-synthetic aporphine, and four naturally occurring aporphines containing methoxyquinoline substructure (nuciferine, D-glaucine, boldine and bulbocapnine). (B) Effects of aporphines on Sm.5HTR$_L$ dependent cAMP generation. HEK293 cells co-transfected with the 22-F cAMP biosensor and Sm.5HTR$_L$ were first treated with the either DMSO vehicle control (open circles) or the indicated compound (solid circles, 5 μM added at solid triangle). After 30 min, 5-HT (0.8 μM, grey triangle) was added. Following stabilization of the 5-HT response, forskolin (20 μM, open triangle) was added to each well. (C) Dose-response curves show inhibition of 5-HT (0.8 μM) evoked cAMP generation in the presence of increasing concentration of individual aporphines.

Table 1
IC$_{50}$ values for aporphine derivatives screened against Sm.5HTR$_L$ and larval schistosomules.

| Compound	IC$_{50}$ Sm.5HTR$_L$ (μM)	IC$_{50}$ schistosomule (μM)
Nuciferine	0.24 ± 0.04	0.62 ± 0.22
D-glaucine	0.86 ± 0.22	3.9 ± 0.72
Boldine	1.1 ± 0.10	2.5 ± 1.4
Apomorphine	15.2 ± 3.3	24.2 ± 15.1
Bulbocapnine	15.2 ± 2.5	76.1 ± 17.8

3. Results

### 3.1. Several aporphinoids act as potent antagonists at Sm.5HTR$_L$

Four commercially available aporphine natural products —

nuciferine, D-glaucine, boldine and bulbocapnine (Fig. 1A) — were screened against recombinantly expressed Sm.5HTR$_L$ in HEK293 cells. Apomorphine, a synthetic aporphine which we have previously shown inhibits schistosomule contractility (Chan et al., 2014), was also examined (Fig. 1A). To profile the activity of Sm.5HTR$_L$, a cAMP-dependent luciferase reporter (pGloSensor-22F) was co-expressed. Sm.5HTR$_L$ is G$_s$ coupled in this system, permitting a rapid and robust read out of 5-HT evoked cAMP generation in live cells (Chan et al., 2016b).

None of the five compounds elevated cAMP levels when administered to Sm.5HTR$_L$ at concentrations ≤100 μM (Fig. 1B). However subsequent addition of 5-HT (0.8 μM) revealed blunted responses to 5-HT in cells treated with the individual aporphine ligands (Fig. 1B), suggesting an antagonist action at Sm.5HTR$_L$. To verify that aporphine-treated treated cells were viable and capable
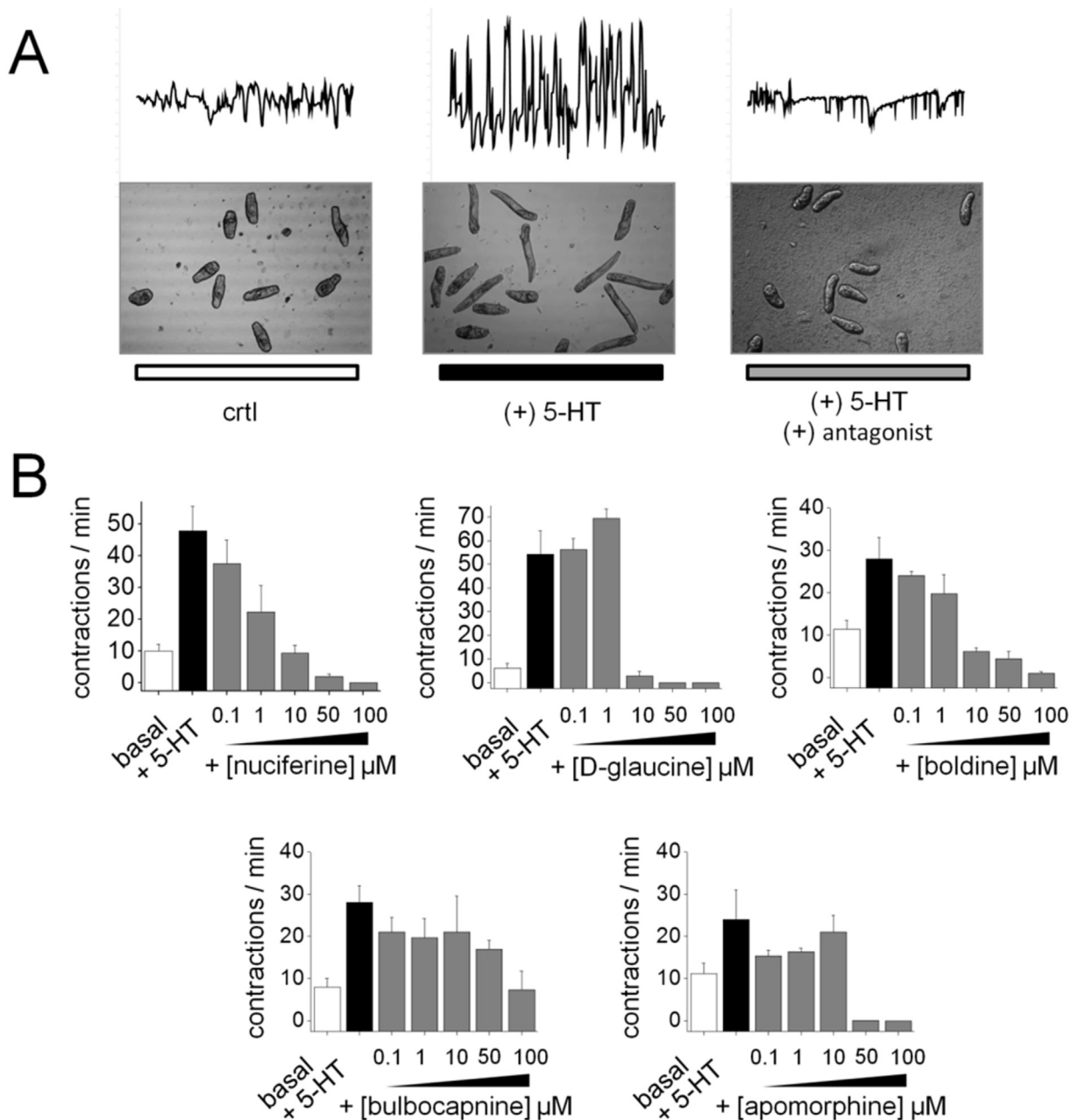
Fig. 2. Aporphine alkaloids inhibit schistosomule contractility. (**A**) Schistosomule body length traces were recorded over 1 min for untreated schistosomules (left), 5-HT (10 μM) treated schistosomules (middle), and schistosomules exposed to both 5-HT and the Sm.5HTR$_L$ antagonist methiothepin (10 μM, right). (**B**) Quantification of body contractions for basal conditions (white bars), addition of 5-HT (10 μM, black bars), and 5-HT plus increasing doses of indicated aporphine (grey bars).
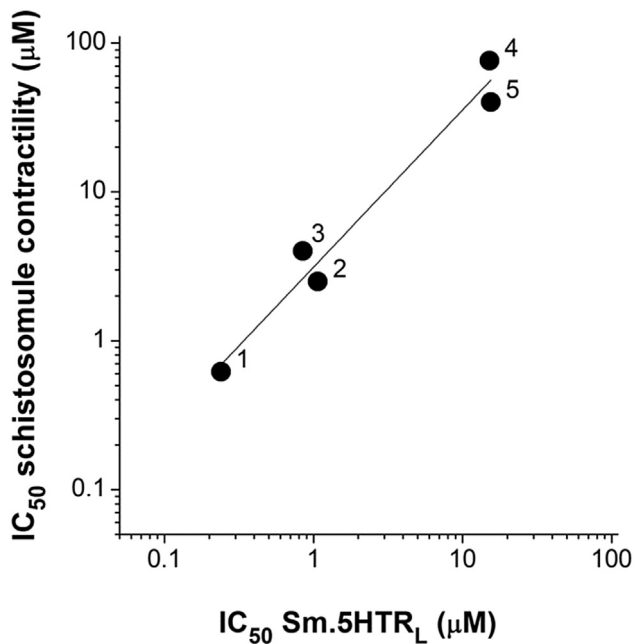
Fig. 3. Correlation of inhibition of Sm.5HTR$_L$ with inhibition of schistosomule mobility. Measured IC$_{50}$ for blockade of Sm.5HTR$_L$ by different aporphine derivatives (ordinate) plotted against measurements of IC$_{50}$ for inhibition of schistosomule contractility (abscissa). Compounds: 1 = nuciferine, 2 = D-glaucine, 3 = boldine, 4 = apomorphine and 5 = bulbocapnine. Data are fitted to a linear equation (y = bx+a), slope = 1.06 ± 0.11 (standard error).

of cAMP generation, forskolin (20 μM) was added to each well. In each case, forskolin elevated cAMP levels to a similar plateau. These data suggest that incubation with aporphine ligands inhibited Sm.5HTR$_L$ dependent cAMP production.

Complete dose inhibition relationships were then performed to assess the potencies of Sm.5HTR$_L$ inhibition. Fig. 1C demonstrates a dose-dependent inhibition of 5-HT-evoked cAMP signaling with IC$_{50}$ values across a ~10-fold range (Table 1). The rank order of potency was nuciferine (IC$_{50}$ = 0.24 μM) < D-glaucine (IC$_{50}$ = 0.86 μM) < boldine (IC$_{50}$ = 1.1 μM) < bulbocapnine(IC$_{50}$ = 15.2 μM) = apomorphine (IC$_{50}$ = 15.2 μM).

3.2. Sm.5HTR$_L$ antagonists ablate schistosomule mobility

Next we screened the aporphine compounds against larval schistosomules. Schistosomules comprise the parasite life cycle stage after penetration of the definitive host by cercariae, and are commonly used in drug screening assays since their periodic body contractions provide a clear phenotypic readout. In this movement assay, exogenous 5-HT stimulates contractility, and knockdown of Sm.5HTR$_L$ (Patocka et al., 2014), or application of the known Sm.5HTR$_L$ antagonist methiothepin (Fig. 2A (Chan et al., 2016b)) reversed this effect.

For quantifying the effects of the aporphine ligands, basal schistosomule movement was first recorded of worms in media lacking 5-HT. 5-HT (10 μM) was then added, resulting in pronounced contractility (~3–5 fold increase), after which increasing doses of Sm.5HTR$_L$ antagonists were added (Fig. 2A). The dose dependent effects of the individual aporphine compounds are shown in Fig. 2B. Nuciferine and D-glaucine were particularly effective at inhibiting larval contractions. Comparison of the potency of these ligands at inhibiting Sm.5HTR$_L$ and larval mobility was then assessed (Fig. 3, Table 1). This correlation demonstrated that the most potent Sm.5HTR$_L$ blockers served as the most

effective inhibitors of schistosomule contraction (Fig. 3, Table 1).

3.3. Effects of aporphine inhibitors of Sm.5HTR$_L$ on adult schistosomes

Next, we screened the three most effective compounds (nuciferine, D-glaucine and boldine, Fig. 3) against cultured adult S. mansoni parasites. Adult S. mansoni also exhibit 5-HT dependent motility which can be measured to quantify drug action (Patocka et al., 2014; Chan et al., 2016b). Adult S. mansoni recovered from infected mice were separated into single sex cohorts and the effects of a fixed concentration of drug (10 μM) on the basal motility of males (Fig. 4A) or females (Fig. 4B) was examined. For males, all drugs impaired basal movement to a similar extent (~50%) compared with control worms (Fig. 4A). For females, nuciferine appeared more effective than the other ligands (p < 0.01 versus p < 0.05, Fig. 4B).

Finally, the effect of ligands on 5-HT stimulated motility was examined. In control worms, 5-HT stimulated movement, most evident in female worms (Fig. 4C&D). In male worms, each aporphinoid decreased responsiveness to increasing concentrations of 5-HT, with worms incubated in nuciferine showing no 5-HT stimulated motility even at the highest 5-HT doses ($\leq$1 mM). Sex-specific difference were observed: with female worms, high doses of 5-HT were competent at restoring motility after D-glaucine or boldine treatment. In contrast, nuciferine again proved to be an effective inhibitor of 5-HT stimulated motility in both female (partial recovery $\geq$100 μM 5-HT) and male worms (nuciferine action not reversed by 5-HT).

4. Discussion

4.1. Target-based discovery of natural products that are Sm.5HTR$_L$ blockers

Natural product based drug discovery has provided critical breakthroughs in the treatment of parasitic disease (Shen, 2015), and there is considerable interest in mining the natural chemical lexicon to identify antischistosomal compounds (Neves et al., 2015). Here, we have studied the action of several aporphine alkaloid natural products (nuciferine, D-glaucine, boldine and bulbocapnine) on 5-HT signaling in schistosomes, which identified nuciferine as an effective inhibitor of GPCR signaling in vitro and 5-HT evoked motility responses in intact parasites.

Nuciferine is one of many aporphine phytochemicals found in the sacred lotus plant (Nelumbo nucifera). The leaves, flowers, seeds and rhizomes of these plants have been broadly used in traditional medicine for treating a spectrum of ailments (Mukherjee et al., 2009). Aporphine compounds isolated from Nelumbo nucifera leaves have been previously shown to have anticestodal profiles, with the mechanistic basis for these effects being ascribed to a free radical scavenging effect (Lin et al., 2014). A more recent study investigating the psychotropic properties of nuciferine demonstrated an affinity for several mammalian serotonergic GPCRs, encompassing action as an inverse agonist at the human 5-HT$_7$ receptor (Hs.5HT$_7$R (Farrell et al., 2016)). Hs.5HT$_7$R is the closest human homolog of Sm.5HTR$_L$, the abundantly expressed schistosome serotonergic GPCR that controls parasite movement (Patocka et al., 2014; Chan et al., 2016b). Furthermore, our recent pharmacological profiling of Sm.5HTR$_L$ identified several compounds with dimethoxyisoquinoline substructure that act as potent and effective Sm.5HTR$_L$ blockers (Chan et al., 2016b). It is therefore notable that the most effective aporphinoid inhibitor of Sm.5HTR$_L$ was nuciferine (contains dimethoxyisoquinoline substructure), followed by two methoxyisolquinoline compounds (D-glaucine and

boldine). The least effective aporphines were bulbocapnine and apomorphine which lacked these moieties (Fig. 1, Table 1). Further exploration of the tolerance of this pharmacophore for serotonergic inhibition and antischistosomal activity is warranted. These results also highlight the importance of optimization of the cAMP biosensor assay for enabling target-based screening of Sm.5HTR$_L$. This methodology has identified leads with comparable potency and penetrance to those generated from larger, more intensive, phenotypic screens. While unbiased, phenotypic screens certainly afford the opportunity for discovering new chemistry that conveys antischistocidal activity, the trade off is often a poorer hit rate (<0.01% (Mansour et al., 2016)).

4.2. Aporphinoid evoked hypomotility correlates with Sm.5HTR$_L$ inhibition

The ability to use the cAMP biosensor to identify Sm.5HTR$_L$ ligands through heterologous expression screening has provided new tools for probing the roles of Sm.5HTR$_L$ in vivo. Sm.5HTR is expressed in (i) the nerves innervating the male body wall, (ii) the male sucker, which is required for anchoring the male-female pair to host vasculature, and (iii) the female caecum, which is involved in the continuous ingestion of red-blood cells from the host circulation (Patocka et al., 2014). These roles for Sm.5HTR are broadly similar to the role reported for the *Caenorhabditis elegans* SER-7 homolog, which regulates neuromuscular signaling to the body wall and feeding behavior (Hobson et al., 2006).

Nuciferine, D-glaucine and boldine completely blocked Sm.5HTR$_L$ dependent cAMP generation in vitro (Fig. 1), and completely ablated schistosome motility (Fig. 2). These data are consistent with the observations of Patocka et al. who used in vivo RNAi methods to demonstrate the key role of Sm.5HTR in supporting larval motility (Patocka et al., 2014). The strong correlation between potency of ligands effecting Sm.5HTR$_L$ blockade and schistosomule inhibition (Fig. 3) underscores the importance of this specific GPCR in regulating motility at this life cycle stage (Patocka et al., 2014). In adults, the ability of Sm.5HTR$_L$ ligands to inhibit basal motility is less (~50%, Fig. 4), possibly indicating the involvement of additional mechanisms governing adult worm muscle activity. Again this result is consistent with in vivo RNAi data (Patocka et al., 2014). Differences in Sm.5HTR$_L$ expression, rates of 5-HT uptake and different drug absorption kinetics between male
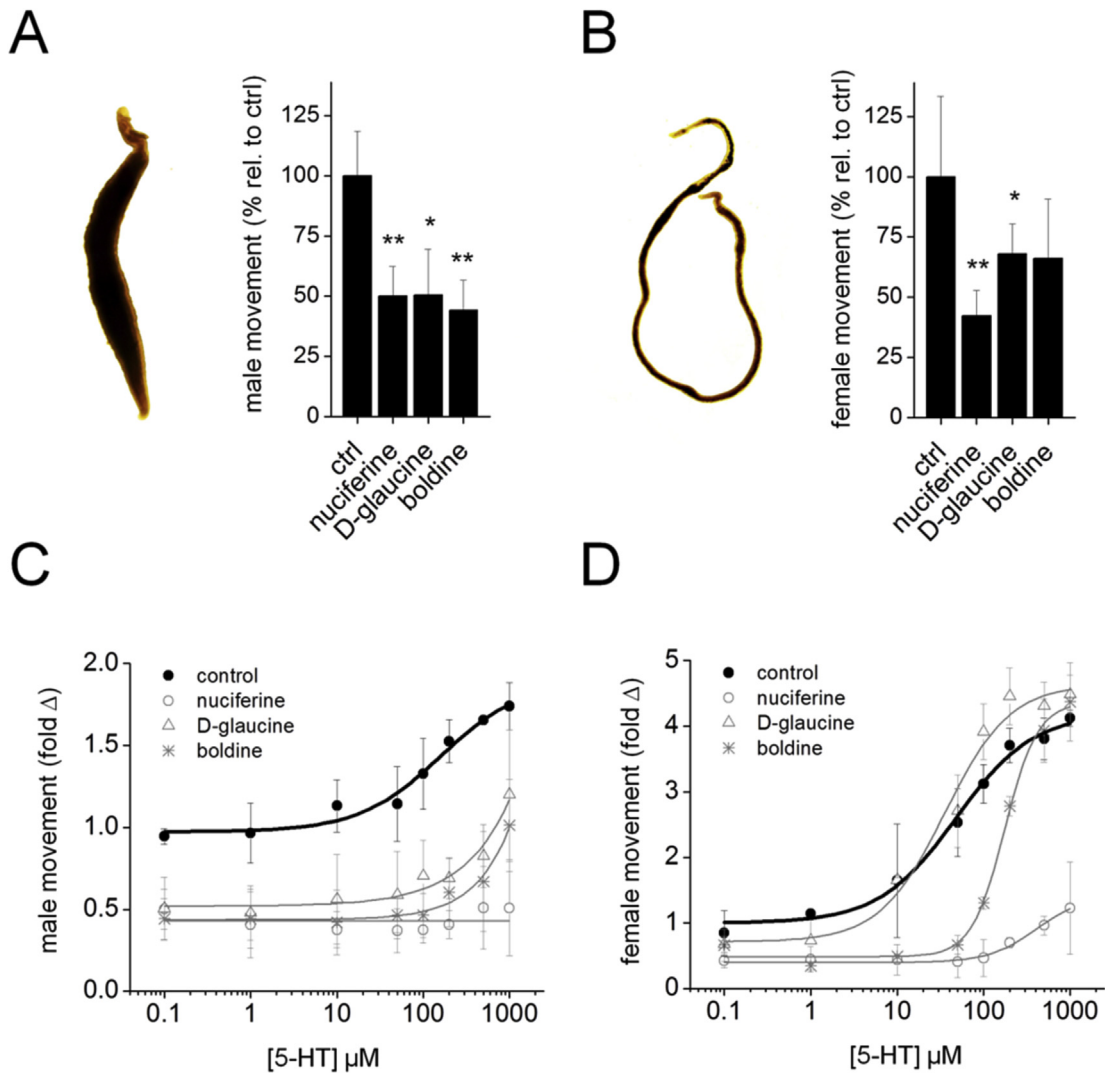

Fig. 4. **Aporphine alkaloid inhibition of adult schistosome movement**. Effects of prioritized compounds on male and female adult schistosome worms. Basal movement of (**A**) male and (**B**) female worms treated with either DMSO ('ctrl') or the indicated compound (10 μM, 2 h). *p < 0.05, **p < 0.01. Effect of aporphine antagonists on 5-HT evoked stimulation of (**C**) male and (**D**) female adult schistosome movement (expressed as fold change over basal movement for DMSO control cohort).

and female adult worms may also contribute to observed differences in effectiveness of individual compounds (Fig. 4). Overall, it is the consistent inhibitory effect of nuciferine against larvae, as well as basal and 5-HT stimulated adult worm motility that is noteworthy. These observations support additional investigation of nuciferine, and related methoxyisoquinoline aporphinoids, as potential anthelmintic agents.

Acknowledgements

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