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In vitro anthelmintic efficacy of inhibitors of phosphoethanolamine Methyltransferases in *Haemonchus contortus*

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ABSTRACT

The essential phosphobase methylation pathway for synthesis of phosphocholine is unique to nematodes, protozoa and plants, and thus an attractive antiparasitic molecular target. Herein, we screened compounds from the National Cancer Institute (Developmental Therapeutics Program Open Chemical Repository) for specific inhibitory activity against *Haemonchus contortus* phosphoethanolamine methyltransferases (HcPMT1 and HcPMT2), and tested candidate compounds for anthelmintic activity against adult and third-stage larvae of *H. contortus*. We identified compound NSC-641296 with IC_{50} values of 8.3 ± 1.1 μM and 5.1 ± 1.8 μM for inhibition of the catalytic activity of HcPMT1 alone and HcPMT1/HcPMT2 combination, respectively. Additionally we identified compound NSC-668394 with inhibitory IC_{50} values of 5.9 ± 0.9 μM and 2.8 ± 0.6 μM for HcPMT1 alone and HcPMT1/HcPMT2 combination, respectively. Of the two compounds, NSC-641296 depicted significant anthelmintic activity against third-stage larvae (IC_{50} = 15 ± 2.9 μM) and adult stages (IC_{50} = 7 ± 2.9 μM) of *H. contortus*, with optimal effective *in vitro* concentrations being 2-fold and 4-fold, respectively, lower than its cytotoxic IC_{50} (29 ± 2.1 μM) in a mammalian cell line. Additionally, we identified two compounds, NSC-158011 and NSC-323241, with low inhibitory activity against the combined activity of HcPMT1 and HcPMT2, but both compounds did not show any anthelmintic activity against *H. contortus*. The identification of NSC-641296 that specifically inhibits a unique biosynthetic pathway in *H. contortus* and has anthelmintic activity against both larval and adult stages of *H. contortus*, provides impetus for the development of urgently needed new efficacious anthelmintics to address the prevailing problem of anthelmintic-resistant *H. contortus*.

1. Introduction

Haemonchus contortus is the most important parasitic nematode of small ruminants that is responsible for substantial economic losses in small ruminant production worldwide (Cantacessi et al., 2012). Anthelmintic drugs have been the only effective method of controlling *H. contortus* infections, but there is now widespread parasite resistance to most of the commercially available drugs (Mortensen et al., 2003; Kaplan, 2004; Gilleard, 2006), thus warranting the urgent need to identify novel molecular targets for developing a new generation of efficacious anthelmintics. Within the infected host, *H. contortus* is a prolific egg layer (Nikolaou and Gasser, 2006), thus requiring active biogenesis of parasite plasma membranes in which phospholipids, particularly phosphatidylcholine, are a major component (Kent, 1995; Vial and Ancelin, 1998).

In the free-living nematode, *Caenorhabditis elegans*, a plant-like phosphobase methylation pathway involving the three-step *S*-adenosylmethionine-dependent methylation of phosphoethanolamine to phosphocholine for the biosynthesis of phosphatidylcholine, has been characterized and found to be essential for *C. elegans* (Palavalli et al., 2006). The phosphobase methylation step in *C. elegans* is catalyzed by two phosphoethanolamine methyltransferases (PMT1 and PMT2), that sequentially methylate phosphoethanolamine to phosphocholine (Palavalli et al., 2006; Brendza et al., 2007). Two enzymes, HcPMT1 and HcPMT2 (similar to the *C. elegans* PMT1 and 2, respectively) have been identified in *H. contortus* and demonstrated to be bonafide phosphoethanolamine methyltransferases for the *S*-adenosylmethionine-

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dependent sequential methylation of phosphoethanolamine to phosphocholine (Lee et al., 2011). Because this phosphobase methylation step does not exist in mammalian cells, it is considered as an attractive molecular target for the establishment of effective parasite-specific therapeutic regimens (Elabbadi et al., 1997; McCarter, 2004; Mitreva et al., 2005).

The *Plasmodium falciparum* phosphoethanolamine methyltransferase (PfPMT) possesses a single methyltransferase domain that methylates all three phosphobases (Pessi et al., 2004, 2005), and has been shown to be essential for the growth and sexual reproduction of the parasite (Witola et al., 2008; Bobenchik et al., 2013). Specific chemical inhibitors for the *P. falciparum* PfPMT catalytic activity have been identified and demonstrated to abrogate parasite gametocyte development as well as parasite asexual development (Bobenchik et al., 2013). These identified inhibitors of PfPMT provide lead chemical entities for development of a new generation of antimalarial drugs. Despite, the identification of *H. contortus* HcPMT1 and HcPMT2, no specific inhibitors for these nematode enzymes have been reported. Herein, we describe the identification of specific inhibitors for the *H. contortus* phosphobase methylation of phosphoethanolamine, and determine the inhibitors' *in vitro* anthelmintic activity against *H. contortus* third-stage larvae and adult worms.

2. Materials and methods

2.1. Isolation of H. contortus worms from goat abomasum

The care and use of goats for experimental procedures in this study was performed following the protocol approved by the University of Illinois at Urbana-Champaign and Tuskegee University Institutional Animal Care and Use Committees. Naturally infected goats with high *H. contortus* egg counts (>5000 eggs per gram of fecal matter) were humanely euthanized and immediately their abomasums were excised and live female *H. contortus* adult worms (identified based on their barber's pole characteristic appearance) were isolated from the abomasal contents by scooping with a spatula and placed in PBS maintained at 37 °C. The worms were washed five times in PBS followed by three washes in RPMI medium (pH 6.8, supplemented with 2% glucose, 500 units/ml penicillin, 500 μg/ml streptomycin, 1.25 μg/ml amphotericin and 1 mM chloramphenicol) (Rhoads and Fetterer, 1995), and finally resuspended in RPMI medium. For culture assays, the parasites in RPMI medium were incubated at 37 °C with 5% CO_2. For RNA extraction, the parasites were kept frozen at −80 °C until use. PCR analysis was performed on genomic DNA extracted from isolated adult worms following the procedure described by Zarlenga et al. (1994) to confirm that they were *H. contortus* and not *Haemonchus placei*.

2.2. Culture and isolation of H. contortus larvae from goat feces

H. contortus larvae were hatched and extracted from goat fecal cultures following the method of Peña et al. (2002). Briefly, fresh fecal samples were collected from the rectum of goats with high *H. contortus* egg counts (>5000 eggs per gram fecal matter) and pooled. About 10 g of the pooled fecal samples were placed in 500 ml beakers and homogenized with an equal volume of vermiculite. About 5 ml of distilled water was added to moisten the homogenate and incubated at 21 °C for 10 days. Every other day, 5 ml of distilled water was added and the homogenate mixed to maintain the moisture content. After 10 days, the third-stage larvae were extracted from the fecal homogenates by the Baermann method. Briefly, the homogenate was re-suspended in about 100 ml of distilled water and applied to a funnel-assembly lined with cheese cloth, with the funnel tip plugged. Additional water was applied to completely fill the funnel assembly. The assembly was left to sit at room temperature overnight to allow the larvae to gravitate through the cheese cloth to the tip of the funnel. The flow-through in which larvae were contained was collected in 50 ml conical tubes and let to settle at 4 °C for 3 h to allow the larvae to sediment. The larvae suspension was washed three times by aspirating out the water (leaving just about 5 ml) followed by addition of 40 ml of autoclaved distilled water (containing 500 units/ml Penicillin G, 500 μg/ml Streptomycin, 1.25 μg/ml Amphotericin and 1 mM Chloramphenicol) and letting stand at 4 °C for 2 h to sediment larvae after which the water was aspirated out leaving only 5 ml. This was followed by washing three times with 40 ml of RPMI-1640 medium (pH 6.8, supplemented with 2% glucose, 500 units/ml penicillin, 500 μg/ml streptomycin, 1.25 μg/ml amphotericin and 1 mM chloramphenicol). The larvae concentration in the final suspension in RPMI-1640 medium was determined by applying 50 μl of the suspension on a glass slide with coverslip and counting using a X10 objective of a light microscope. Larvae re-suspended in medium were maintained in culture at 37 °C, 5% CO_2. PCR analysis was performed on genomic DNA extracted from isolated larvae following the procedure described by Zarlenga et al. (1994) to confirm that they were *H. contortus* and not *H. placei*.

2.3. H. contortus cDNA synthesis and analysis of HcPMT1 and HcPMT2 expression

About 150 mg of pelleted *H. contortus* female adult worms were homogenized in 1 ml of Trizol reagent using a mortar and pestle and total RNA was extracted from the homogenate following the Trizol reagent protocol (Life Technologies). One microgram of total RNA was treated with DNase I (Invitrogen) to remove residual genomic DNA and reverse transcription (RT) was performed using the iScript Select cDNA Synthesis kit (BIO-RAD) following the kit protocol. Quantitative real time PCR to determine transcript levels for HcPMT1 and HcPMT2 was performed using the *H. contortus* cDNA as template. The HcPMT1 primer pair used was 5′-TTCACTACTGTGCTAGCA-3′ (forward) and 5′-CTTTCAATGACCACGAT-3′ (reverse), while the HcPMT2 primer pair was 5′-ATGCCCTCAGGATGACCAGAGAA-3′ (forward) and 5′-GGATCGTAGAGCAGCAGTGATAAA-3′ (reverse). The primer pair for *H. contortus* β-tubulin (Genbank accession number: EF198865) was 5′-CTTGTTGATCTCGAGCCTGGAA-3′ (forward) and 5′-AAGGCAATCACAACCTTCAGCTT-3′ (reverse). To generate quantification standards, PCR products for each primer pair were fractionated on agarose gel and extracted by the QIAquick® Gel extraction kit (Qiagen). The extracted gene fragments were quantified by Nanodrop Spectrophotometer (Fisher) followed by 10-fold serial dilution to prepare quantification standards. Real-time PCR mix consisted of 1 μl cDNA as template, 1 μl of primer mix (500 nM each), and 10 μl of SsoFast EvaGreen supermix (Bio-Rad), with the final volume made up to 20 μl with nuclease-free water. Cycling was performed using a CFX 96 real-time system (Bio-Rad), and transcript quantities were derived by the system software using the generated standard curves. The relative amounts of HcPMT1 and HcPMT2 transcripts were derived by dividing their respective concentrations by the concentration of β-tubulin.

2.4. Cloning of HcPMT1 and HCPMT2 coding sequences

Primer pairs for amplification of the coding sequences of HcPMT1 and 2 were designed based on the gene sequences reported by Lee et al. (2011). The primer pair for HcPMT1 coding sequence was 5′-*CTCGAG***ATG**ACGGCTGAGGTGCGACGGGATT-3′ (Forward, with the *XhoI* restriction site italicized and start codon in bold) and 5′-*GGATCC***TTA**AAGTGAAGCCTTGATCA-3′ (Reverse, with the *BamHI* site italicized and stop codon in bold). The primer pair