International Journal for Parasitology: Drugs and Drug Resistance 6 (2016) 44–53

Contents lists available at ScienceDirect

International Journal for Parasitology: Drugs and Drug Resistance

journal homepage: www.elsevier.com/locate/ijppaw

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

William H. Witola [a, *], Kwame Matthews [b], Mark McHugh [b]

[a] Department of Pathobiology, College of Veterinary Medicine, University of Illinois, Urbana-Champaign, USA
[b] Department of Agricultural and Environmental Sciences, Tuskegee University, Tuskegee, AL, USA

ARTICLE INFO

Article history:
Received 28 November 2015
Received in revised form
12 January 2016
Accepted 12 January 2016
Available online 18 January 2016

Keywords:
Haemonchus contortus
Phosphoethanolamine *N*-methyltransferase
Phosphatidylcholine
Chemical inhibitors
Anthelmintic activity

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-

* Corresponding author. Department of Pathobiology, College of Veterinary Medicine, University of Illinois, Urbana-Champaign, 2001 South Lincoln Avenue, Urbana, IL 61802, USA.
E-mail address: whwit35@illinois.edu (W.H. Witola).

http://dx.doi.org/10.1016/j.ijpddr.2016.01.002

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

for HcPMT2 coding sequence was 5′-*CTCGA-G***ATG**CCTGCCGTTGAACGACAACTGATT-3′ (Forward, with the *XhoI* restriction site italicized and start codon in bold) and 5′-*GGATCC***TTA**TTGTGGCTTGACAGCAGCGAA-3′ (Reverse, with the *BamHI* site italicized and stop codon in bold). The forward and reverse primers incorporated the *XhoI* and *BamHI* restriction sequences, respectively, for cloning at the *XhoI*/*BamHI* unique site of pET15b vector (Novagen). The genes were amplified by polymerase chain reaction from *H. contortus* cDNA using high fidelity DNA polymerase (Affymetrix) and cloned into pGEMT for sequencing to confirm identity. The genes were excised from the pGEMT vector by dual *XhoI*/*BamHI* restriction enzyme digestion and sub-cloned at the *XhoI*/*BamHI* site in the pET15b expression vector in-frame with the hexahistidine (His-tag) at the N-terminal. The recombinant vectors were propagated in DH5α *E. coli* and subsequently transformed into protein expression *E. coli* BL21-CodonPlus-DE3-RIL (Stratagene).

2.5. Expression and purification of recombinant HcPMT1 and HcPMT2

E. coli BL21-CodonPlus-DE3-RIL strain transformed with pET15b carrying either HcPMT1 or HcPMT2 coding sequence were cultured at 37 °C in Luria broth medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol to an A_{600} of 0.6 and protein expression induced by the addition of 1 mM isopropyl-β-d-thiogalactopyranoside. Bacteria was harvested from the cultures by centrifugation and resuspended in BugBuster reagent for native protein extraction (Novagen) containing a protease inhibitor mix (Roche Diagnostics), 40 units/ml benzonase and 10 μg/ml lysozyme. The resuspended bacteria was lysed by sonication on ice and the cleared soluble fraction of the lysate separated by centrifugation. The His-tagged proteins were purified from the soluble fraction by nickel-affinity chromatography according to the manufacturer's instructions (Novagen). The eluates were dialyzed using a buffer containing 5 mM Hepes–KOH (pH 7.8) and 0.5 mM DTT. The purity of the recombinant proteins was determined by SDS/PAGE, and the protein concentration was measured by the method of Bradford using BSA as a standard.

2.6. Optimization of enzyme, substrate and co-substrate concentrations in Methyltransferase assay

The Methyltransferase HT activity kit (ENZO) is a fluorescent assay for the determination of *S*-adenosyl-L-methionine-dependent methyltransferase activity. It is ideal for high throughput screening of candidate compounds that may alter the activity of *S*-adenosylmethionine (SAM)-dependent methyltransferases. We customized the Methyltransferase HT Activity kit assay for the use of optimal HcPMT1 and HcPMT2 proteins as enzymes, SAM as the methyl-donor, and phosphoethanolamine (PE) as the substrate. To test the enzymatic activity of HcPMT1 and HcPMT2 using the Methyltransferase HT Activity kit, SAM was initially used at the kit's recommended starting concentration of 80 μM, while PE was used at 160 μM (twice as much as SAM). The concentration of HcPMT1 and HcPMT2 were titrated either individually or in combination to determine the optimal concentration for catalyzing the methylation of PE following the kit's instructions. Briefly, the concentrated HcPMT1 and HcPMT2 protein solutions, SAM and PE were diluted in the kit's 1X Transferase Assay buffer and added to a reaction mixture containing 25 μl 1X Methyltransferase Reaction Buffer and 100 μl of Detection Solution, and the final volume made up to 150 μl with 1X Transferase Assay buffer. The final concentration of each of SAM and PE in the reaction mixture was 80 μM and 160 μM, respectively, while the final concentration of HcPMT1 and HcPMT2 proteins varied in increment. A blank reaction mixture lacked protein and its fluorescence was used for correcting for background fluorescence in the test samples. The reactions were prepared in triplicate wells in a black flat-bottomed 96 well plate, covered with a foil plate sealer and incubated at room temperature (24 °C) with shaking for 45 min. After 45 min of incubation, a positive reaction mixture was prepared containing 25 μl of 1X Positive Control solution, 100 μl Detection solution and 25 μl of 1X Methyltransferase Reaction Buffer, and the plate incubated for an additional 20 min with shaking at room temperature. Then, 50 μl of ice-cold isopropanol was added to each reaction mixture to stop the reaction and the fluorescence read at 380ex/520em using a Gemini XPS Microplate fluorescence reader (Molecular Devices).

To determine the optimal concentration of PE in the assay, increasing concentrations of PE (starting at 40 μM) were used, with the concentration of SAM maintained at 80 μM and HcPMT1 and HcPMT2 maintained at the derived optimal concentrations. Likewise, to determine the optimal concentration of SAM in the assay, increasing concentrations of SAM (starting at 40 μM) were used with the concentration of PE, HcPMT1 and HcPMT2 maintained at the derived optimal concentrations. To determine the substrate specificity of HcMPT1 and HcPMT2 enzymes, ethanolamine and phosphatidylethanolamine were each used in the methyltransferase assay at 160 μM as alternative substrate with SAM as co-substrate at 80 μM.

2.7. Screening chemical compounds for inhibitory activity against HcPMT1 and HcPMT2

The chemical compounds were obtained from the National Cancer Institute/Developmental Therapeutics Program Open Chemical Repository. The compounds were reconstituted in dimethyl sulfoxide (DMSO) as stock solutions. Just before use, aliquots of the stock solutions were diluted in the Methyltransferase HT kit's 1X Transferase Assay Buffer and added at increasing concentrations to the reaction mix (containing 25 μl 1X Methyltransferase Reaction Buffer, 100 μl of Detection Solution, 95 μM SAM, 160 μM phosphoethanolamine, 11 μg/ml each of HcPMT1 and HcPMT2) and the final volume made up to 150 μl with 1X Transferase Assay buffer. Reaction mixtures without inhibitor, but containing DMSO (equivalent to the volume of inhibitor stock solution used in the test reactions) were set up as the non-inhibited reactions. A blank reaction mix did not contain inhibitor nor did it contain HcPMT1 and HcPMT2. All the reactions were prepared in triplicate wells in a black flat-bottomed 96 well plate, covered with a foil plate sealer and incubated at room temperature (24 °C) with shaking for 45 min. After 45 min of incubation, a positive reaction mixture was prepared containing 25 μl of 1X Positive Control solution, 100 μl Detection solution and 25 μl of 1X Methyltransferase Reaction Buffer, and the plate incubated for an additional 10 min with shaking at room temperature (24 °C). Then, 50 μl of iced-cold isopropanol was added to each reaction to stop the reaction and the fluorescence read at 380ex/520em. The percent inhibition of each compound dilution was calculated using the following formula:
where:

$$\text{Percent Inhibition} = \frac{(\text{Mean non-inhibited reaction RFU} - \text{Mean Inhibited reaction RFU}) \times 100}{\text{Mean non-inhibited reaction RFU}}$$

- Mean non-inhibited reaction RFU is the average relative fluorescence (RFU) for triplicate assays of the reaction assay without inhibitor.
- Mean inhibited reaction RFU is the average RFU for triplicate assays of the reaction assay with inhibitor.

The IC_{50} values for the inhibitory activity of the compounds were derived using nonlinear regression analysis by Graph pad Prism software version 6.0 program.

2.8. Chemical compound cytotoxicity assay

To determine the cytotoxic concentrations of the candidate compounds in mammalian cells, a colorimetric assay using the cell proliferation reagent WST-1 (Roche) for the quantification of cell viability was performed on MonoMac6 cells (Ziegler-Heitbrock et al., 1988). MonoMac6 cells were seeded at 10^4/well in RPMI-1640 medium (without red phenol) supplemented with 10% fetal calf serum, 2.05 mM L-glutamine, 1 × nonessential amino acids (Sigma), OPI medium supplement (Hybri Max; Sigma), and 1% penicillin-streptomycin in 96 well plates. Each candidate compound was added to triplicate wells at increasing concentrations. Control wells without chemical compound, but to which equivalent volumes of DMSO (chemical compound solvent) was added were also set up. After 48 h of culture at 37 °C, with 5% CO_2, a colorimetric assay using the cell proliferation reagent WST-1 (Roche) for the quantification of cell viability was performed on the cultures by adding 20 μl of the WST-1 reagent to each well. After mixing, the plates were wrapped in aluminum foil and incubated for 1 h at 37 °C with 5% CO_2. After 1 h of incubation, 150 μl of the medium from each well was transferred to a new 96-well plate and quantification of the formazan dye produced by metabolically active cells was read as absorbance at a wavelength of 420 nm using a scanning multi-well spectrophotometer (Spectra Max 250; Molecular Devices).

2.9. Analysis of the anthelmintic effect of chemical compounds

One hundred adult *H. contortus* female worms (freshly isolated from the abomasum of goat) or 100 *H. contortus* third-stage (L3) larvae (freshly isolated from fecal cultures) were resuspended in 20 ml of supplemented RPMI-1640 medium (containing 500 units/ml Penicillin G, 500 μg/ml Streptomycin, 1.25 μg/ml Amphotericin and 1 mM Chloramphenicol) in petri dishes in triplicate with or without increasing concentrations of candidate chemical compound, and incubated at 37 °C with 5% CO_2 for 36 h. To control cultures without chemical compound, equivalent volumes of DMSO (chemical compound solvent) was added. Additionally, control cultures without DMSO nor compound were included. After 36 h of culture, the petri dishes were placed under bright fluorescent light for 5 min on a heat pad maintained at 37 °C to stimulate the worms, and then 50 worms in each dish were randomly counted using a dissection light microscope. Of the 50 worms counted, the percentages of very sinuously motile, sluggishly motile (intermittent jerky movements) and immotile worms were determined. Based on the percentage of the non-motile parasites at each concentration of the compound tested, the IC_{50} of the compound was derived using nonlinear regression analysis by Graph pad Prism software version 6.0 program.

2.10. Statistical analysis

Statistical analyses were performed by analysis of variance (ANOVA) using the GLM Procedure of SAS (SAS Institute, Cary, NC) and the significance level was set at $P < 0.05$.

3. Results

3.1. Expression analysis and cloning of HcPMT1 and HcPMT2

The HcPMT1 coding sequence we amplified from *H. contortus* cDNA was found to be 1050 bp, which when aligned with the HcPMT1 sequence reported by Lee et al. (2011), lacked a 333 bp fragment positioned at 859 bp to 1192 bp of the coding sequence reported by Lee et al. (2011) (Fig. 1), suggesting that the extra 333 bp fragment in the sequence of Lee et al. (2011) could be an intron. Despite the difference, alignment of the amino acids sequences showed that the two genes coded for proteins encompassing an identical N-terminal methyltransferase domain, indicating that they both would retain their functionality. The sequence we amplified and cloned for HcPMT2 was identical to that reported by Lee et al. (2011). Using the *H. contortus* cDNA, we performed quantitative real time PCR to determine the expression levels of HcPMT1 and HcPMT2 normalized to *H. contortus* β-tubulin. HcPMT1 transcripts were 1.2-fold higher than HcPMT2 transcripts, but there was no significant ($P < 0.05$) between the two.

3.2. HcPMT1 and HcPMT2 catalyze the SAM-dependent methylation of phosphoethanolamine

To analyze the activity of HcPMT1 and HcPMT2 proteins in catalyzing the SAM-dependent trans-methylation of phosphoethanolamine (PE) using the Methyltransferase HT Activity kit assay (ENZO), HcPMT1 and HcPMT2 were expressed and purified natively (Fig. 2) and used in the assay as enzymes. First, we tested the individual proteins' catalytic activity at varying concentrations and found that HcPMT1 alone depicted a concentration-dependent increase in catalytic activity while HcPMT2 on its own did not show any significant catalytic activity at all the concentrations tested. However, when HcPMT1 and HcPMT2 were combined the catalytic activity was significantly higher ($P < 0.05$) than that of HcPMT1 alone in a concentration-dependent manner (Fig. 3). During the SAM-dependent methylation of PE, HcPMT1 catalyzes the first step leading to the formation of phosphomonomethylethanolamine (PMME) and the release of *S*-adenosylhomocysteine (SAH) (Lee et al., 2011). The SAH activates the fluorophore in the kit's detection solution leading to detectable fluorescence. HcPMT2 catalyzes the methylation of PMME to phosphodimethylethanolamine (PDME) and subsequent methylation of PDME to phosphocholine, releasing SAH at each methylation step. This is consistent with the observed lack of catalytic activity when HcPMT2 is used as the sole enzyme in the assay because the only substrate in the reaction mixture was PE which is not a substrate for HcPMT2. On the other hand, when HcPMT1 is used alone it's able to methylate PE, with the activity increasing significantly when both HcPMT1 and HcPMT2 are added to the assay because this results in the generation of PMME which is a substrate for HcPMT2. The optimal concentration of HcPMT1 was found to be about 11 μg/ml (Fig. 3A). Similarly when HcPMT1 and HcPMT2 were used in combination, the optimal concentration of each protein in the assay was about 11 μg/ml and this was the concentration that was used in the subsequent assays. Titration of the concentration of PE and SAM depicted optimal concentrations of 160 μM and 95 μM, respectively (Fig. 3B and C) and these were the concentrations that were used in the subsequent assays. To determine the substrate specificity of combined activity of HcPMT1 and HcPMT2, ethanolamine or phosphatidylethanolamine were used as substrate with SAM as co-substrate. At all concentrations tested, there was no notable significant methylation activity for both ethanolamine and phosphatidylethanolamine (data not shown).

```
WW   ATGACGGCTGAGGTGCGACGGGATTCCTTCAAGACGTTCTGGGACAAGTACTCAGATAAACCCGACACTAATTCGATGATGCTCAACCAGACTGCACAA 99
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
LS   ATGACGGCTGAGGTGCGACGGGATTCCTTCAAGACGTTCTGGGACAAGTACTCAGATAAACCCGACACTAATTCGATGATGCTCAACCAGACTGCACAA 99

WW   GATCTGGAAGCTAGCGATAGAGCAGATATCCTCTCCAGCCTACCTCACCTAACCAACAAAGACGTGGTCGATATTGGCGCTGGAATCGGGCGCTTCACT 198
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
LS   GATCTGGAAGCTAGCGATAGAGCAGATATCCTCTCCAGCCTACCTCACCTAACCAACAAAGACGTGGTCGATATTGGCGCTGGAATCGGGCGCTTCACT 198

WW   ACTGTGCTAGCAGAAACTGCTCGATGGGTTCTTTCAACGGATTTCATCGAATCGTTCATCGAAAAAAATCAAGAACGAAATGCTCACATGGGTAACATC 297
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
LS   ACTGTGCTAGCAGAAACTGCTCGATGGGTTCTTTCAACGGATTTCATCGAATCGTTCATCGAAAAAAATCAAGAACGAAATGCTCACATGGGTAACATC 297

WW   AGTTATCAAATAGGAGACGCAGTCCATTTGCAAATGGACGAGAAAAGCGTGGATCTCGTTTTTACGAATTGGTTGATGATGTATCTCTCCGATCGTGAA 396
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
LS   AGTTATCAAATAGGAGACGCAGTCCATTTGCAAATGGACGAGAAAAGCGTGGATCTCGTTTTTACGAATTGGTTGATGATGTATCTCTCCGATCGTGAA 396

WW   GTCATTGAATTTCTGCTGAATGCTATGCGATGGTTGAGAGCGGACGGATACATTCATCTCAGAGAAAGCTGCTCCGAGCCAAGCACGGGCCGTCTGAAG 495
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
LS   GTCATTGAATTTCTGCTGAATGCTATGCGATGGTTGAGAGCGGACGGATACATTCATCTCAGAGAAAGCTGCTCCGAGCCAAGCACGGGCCGTCTGAAG 495

WW   ACCGCCACAATGCACTCAGCCGTTGACGCCAACCCAACACATTACCGTTTCTCATCGCTGTATATCAAGCTTCTTCGAGCAATCCGATACGGGGACAGT 594
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
LS   ACCGCCACAATGCACTCAGCCGTTGACGCCAACCCAACACATTACCGTTTCTCATCGCTGTATATCAAGCTTCTTCGAGCAATCCGATACGGGGACAGT 594

WW   GATGGAAAAATGTGGAAATTTGATGTGCAGTGGAGCTGCTCGGTGCCCACCTACATACGGAGGTGCAATAACTGGCGTCAAGTGCATTGGTTGACGAAG 693
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
LS   GATGGAAAAATGTGGAAATTTGATGTGCAGTGGAGCTGCTCGGTGCCCACCTACATACGGAGGTGCAATAACTGGCGTCAAGTGCATTGGTTGACGAAG 693

WW   AAGGTACCGGCAGTTGGCGACGAAGAGACTTCAGTCGACGATTTGCTCAACTTGTTCAGCCAGATCTGGCCAGCCGAACAAAAGACGTGGGATGAAAAA 792
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
LS   AAGGTACCGGCAGTTGGCGACGAAGAGACTTCAGTCGACGATTTGCTCAACTTGTTCAGCCAGATCTGGCCAGCCGAACAAAAGACGTGGGATGAAAAA 792

WW   CTAGACAATGAAAAATACAGTTGGACTGATAAGATATTCTCGAATGCGATCGATGATGAAGTGGTGC---------------------------------- 859
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
LS   CTAGACAATGAAAAATACAGTTGGACTGATAAGATATTCTCGAATGCGATCGATGATGAAGTGGTGCCAAAGAACAGTACCGCCTATGTCTTCACACCA 891

WW   ---------------------------------------------------------------------------------------------------- 859

LS   AGGCAACGATCCCCCTTCTTGCACGTCAACTCGCACCTTTTGGCAGAGAAGTTCACATGCAATGTATGGAATGTTGAAACAAAAGAGTATTTGTATCGT 990

WW   ---------------------------------------------------------------------------------------------------- 859

LS   ACTTCGTTGACGAAGGCAAACAACCAGAAGGACCAACGAGTGCGCTTCGGTTGGAACGAGTCCTTGTCTTCGCCCATCGACTACTGGAATCAGAGGGAC 1089

WW   ---------------------------------------------------------------------------------------------------- 859

LS   GCTTCATTTGACTGCATGGTAGCAACTGAACTTCTCGCGACTTGTGATGATGAGAGCGTAAAGAGTATTGCGAGCATTATGAAACCAGAAGCGAAGGTG 1188

WW   ----TCCTCGAACCAGTTAGCGGAATTGACGAGACGTCCGTTAGGCAGCGAATGACTACTTGTGGGTTCAAAAACATTACCATCGTCGATGTTACACAG 954
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
LS   GTGCTCCTCGAACCAGTTAGCGGAATTGACGAGACGTCCGTTAGGCAGCGAATGACTACTTGTGGGTTCAAAAACATTACCATCGTCGATGTTACACAG 1287

WW   GAGTCCTTGAACGCCGAGGTTTCTTTCATTAAGGACCACAACTTGGACGTCGAACTCTCTGGTTGTAATTACCTACTGATCAAGGCTTCACTTTAA    1050
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
LS   GAGTCCTTGAACGCCGAGGTTTCTTTCATTAAGGACCACAACTTGGACGTCGAACTCTCTGGTTGTAATTACCTACTGATCAAGGCTTCACTTTAA    1383
```

Fig. 1. Alignment of *H. contortus* HcPMT1 coding sequence cloned in the present study (**WW**) with the sequence reported by Lee et al. (2011) (**LS**). The region indicated by dashed line (—) illustrates the sequence fragment that is present in the LS but absent in the WW coding sequence.

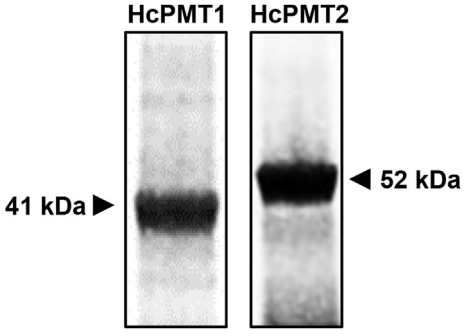

Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of affinity column chromatography-purified recombinant His-tagged HcPMT1 and HcPMT2 proteins. HcPMT1 and HcPMT2 were expressed as 41 kDa and 52 kDa recombinant proteins, respectively.

3.3. Analysis of compounds' inhibitory effect on catalytic activity of HcPMT1 and HcPMT2

To identify chemical compounds that have inhibitory activity against the methyltransferase activity of HcPMT1 and HcPMT2, we obtained 28 chemical compounds (Table 1) from the National Cancer Institute Open Chemical Repository that we had previously tested for inhibitory activity against the *P. falciparum* phosphoethanolamine methyltransferase (PfPMT) (Bobenchik et al., 2013). Among these 28 compounds we found two compounds, NSC-641296 and NSC-668394, that inhibited the catalytic activity of HcPMT1 as the sole enzyme and the combination of HcPMT1 and HcPMT2 in a concentration-dependent manner (Fig. 4A and B). The inhibitory effects of NSC-641296 and NSC-668394 against the combination of HcPMT1 and HcPMT2 were significantly ($P < 0.05$) higher than that of HcPMT1 as the sole enzyme at all concentrations tested suggesting that these two compounds were inhibiting

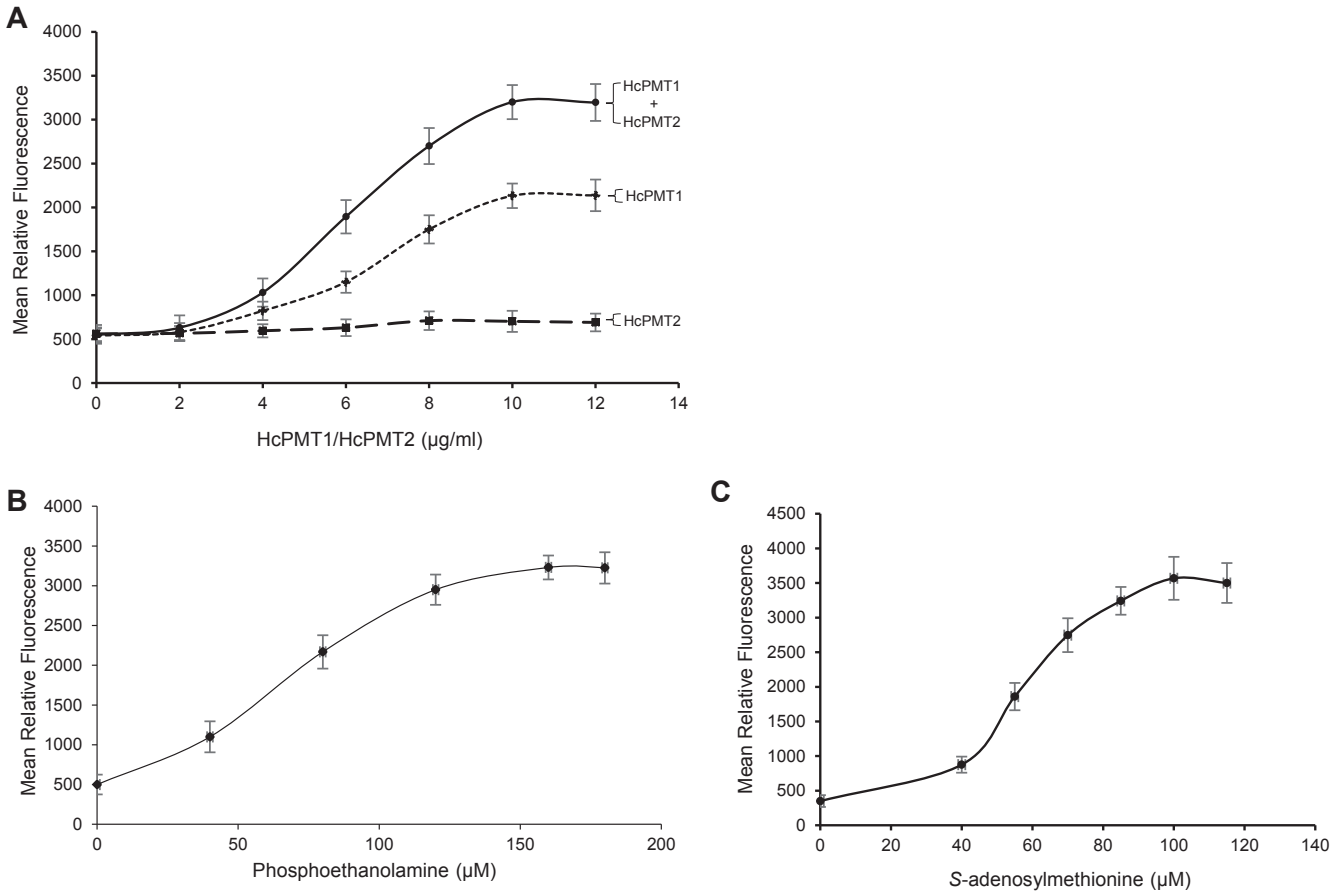

Fig. 3. (A) Titration of HcPMT1 only (dotted line), HcPMT2 only (dashed line) and HcPMT1 + HcPMT2 (solid line) in the methyltransferase assay with *S*-adenosylmethionine and phosphoethanolamine each maintained at 80 μM and 160 μM, respectively. When HcPMT1 and HcPMT2 were used in combination, each was added at the depicted concentration. (B) Titration of phosphoethanolamine concentration in the methyltransferase assay with *S*-adenosylmethionine maintained at 80 μM and each of HcPMT1 and HcPMT2 maintained at 11 μg/ml in the reaction mixtures. (C) Titration of *S*-adenosylmethionine with phosphoethanolamine maintained at 160 μM and each of HcPMT1 and HcPMT2 maintained at 11 μg/ml in the reaction mixtures. The data shown represent means of three independent experiments with standard error bars.

the activities of both HcPMT1 and HcPMT2 (Fig. 4A and B). NSC-641296's IC_{50} values were 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 (Table 2). NSC-668394's inhibitory IC_{50} values were 5.9 ± 0.9 μM and 2.8 ± 0.6 μM for HcPMT1 alone and HcPMT1/HcPMT2 combination, respectively (Table 2). Compounds NSC-158011 and NSC-323241 were observed to have modest inhibitory effect against the catalytic activity of the combination of HcPMT1 and HcPMT2 and did not have any significant inhibitory effect against the activity of HcPMT1 as the sole enzyme (Fig. 4C and D), suggesting that they only inhibited the activity of HcPMT2. Compound NSC-158011 inhibited the combined activity of HcPMT1 and HcPMT2 to a maximum of 12% at a concentration of about 15 μM (Fig. 4C), while the maximum inhibition rate (21%) for compound NSC-323241 was attained at a concentration of 12 μM (Fig. 4D). DMSO only at all volumes used did not affect the catalytic activity of the enzymatic assays without inhibitors (data not shown).

3.4. Anthelmintic effect of compounds with inhibitory effect against HcPMT1 and HcPMT2

To determine the anthelmintic effect of the chemical compounds that had inhibitory effect against the catalytic activity of HcPMT1 and HcPMT2, we cultured *H. contortus* adult female worms and third-stage larval worms in the presence or absence of increasing concentrations of compounds NSC-641296, NSC-668394, NSC-158011 and NSC-323241. After 36 h of culture, a total of 50 worms per culture were randomly counted to establish the percentage of worms that were actively sinuously motile, sluggishly motile or non-motile. For all compound assays, analysis of worm counts in the control cultures with or without increasing concentrations of DMSO showed that DMSO (up to the highest amount used in the assays) had no effect on the motility of the worms.

In our preliminary work, we had observed that the effect of compound NSC-641296 on motility of *H. contortus* was notable as early as 24 h of culture, and became more evident at 36 h of culture. Therefore, considering that culture of *H. contortus* is transient, with the associated risk of fungi and bacteria proliferation if cultures are maintained for a long time, we chose to analyze the assays at the earliest time point (36 h of culture) when the effect of the compound NSC-641296 was prominently evident. In larval cultures treated with compound NSC-641296, there was a compound concentration-dependent reduction in larval motility when compared to the untreated larvae. As shown in Fig. 5, a significant ($P < 0.05$) decrease in the percentage of very motile larvae and a corresponding significant ($P < 0.05$) increase in sluggishly motile larvae was observed starting at 4 μM of NSC-641296 in medium. Above 4 μM of NSC-641296, the percentage of sluggishly motile larvae was at least 2.7-fold significantly ($P < 0.001$) higher than that of very motile worms. From 14 μM and above of NSC-641296, the

Table 1
Chemical compounds screened for inhibitory activity against the catalytic activity of HcPMT1 and HcPMT1 based on the list of compounds reported by Bobenchik et al. (2013).

NSC#	Chemical formula	Chemical name
7346	C10H12CuN2O8.2Na	Cuprate(2-), [[N,N′-1,2-ethanediylbis[N-(carboxymethyl)glycinato]](4-)-N,N′,O,O′,ON,ON′]-, disodium, (OC-6-21)
22225	C9H4Cl6O4.Cd	1,2,3,4,7,7-hexachlorobicyclo[2.2.1]hept-2-ene-5,6-dicarboxylic acid
24048	C21H18N2O.HI	**ND**
37031	C20H14N2O7S2	**ND**
39225	C12H20N2O2	1,2-Benzenediol,3,5-bis[(dimethylamino)methyl]
46613	C13H13N5	**ND**
47924	C18H17NO2	**ND**
57998	C13H19NS.C7H4ClNO3	**ND**
85459	C16H24N2O4	3,6-Bis(morpholinomethyl)pyrocatechol
88947	C17H13NO8S2.Na	**ND**
109268	C32H36Cl2Cu2N2O2	Copper, di.mu.-chloribis[1-[(1-piperdinyl-.kappa.N)methyl]-2-naphthalenolato-.kappa.O]di-
113997	C14H10O4S2	**ND**
125034	C18H12N2O2S2	2,2′-disulfanediyldiquinolin-8-ol
150080	C12H17N3O3	2-(1,3-dimethylhexahydropyrimidin-2-yl)-4-nitrophenol
158011	C18H15NS2	N-naphthalen-1-yl-2-phenylsulfanylethanethioamid
169942	C16H28Cu2N8O8	**ND**
173904	C24H29ClN2O4	Carbamic acid, [1-[[[3-chloro-2-oxo-1-(phenylmethyl)propyl]amino]carbonyl]-3-methylbutyl]-, phenylmethyl ester
175493	C9H11FeNOS2	Iron, carbonyl(.eta.(5)-2,4-cyclopentadien-1-yl) (dimethylcarbamodithioato-S,S′)-
310551	C18H20CuN6S4	Copper; [(6-methylpyridin-2-yl)methylideneamino]-(methylsulfanylsulfoniumylidenemethyl)azanide
323241	C16H22N4Se	3-Azabicyclo[3.2.2]nonane-3-carboselenoic acid, [1-(2-pyridinyl)ethylidene]hydrazide
348401	C11H6N8O3S	4-(6-Thioguanine)-7-nitro-2,1,3-benzoxadiazole
371777	C15H14N2O4S	**ND**
622648	C8H13N2S3.ClO4	**ND**
632233	C16H15NO2S	**ND**
638646	C26H24Cl4N2O3.ClH	4-Piperidinone, 3,5-bis[(3,4-dichlorophenyl)methylene]- 1-[3-(4-morpholinyl)-1-oxopropyl]-, monohydrochloride
641296	C13H19ClCuN4S	Hydrazinecarbothioamide, N,N-dipropyl- 2-(2-pyridinemethylene)-, (N,N,S)-copper(II) chloride complex, (SP-4-3)-
668394	C17H12Br2N2O3	5,8-Quinolinedione, 7-[[2-(3,5-dibromo-4-hydroxyphenyl)ethyl]amino]-

ND, not derived.

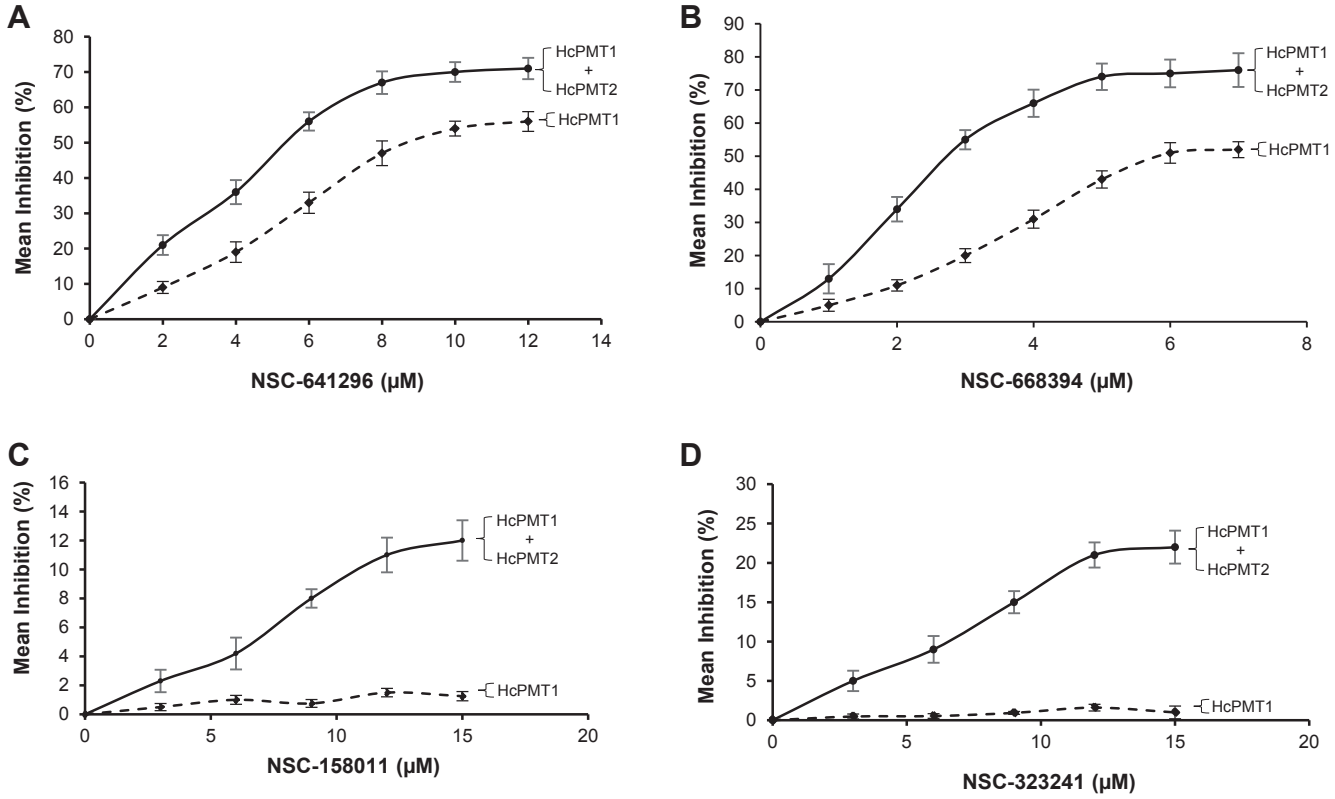

Fig. 4. Percent inhibitory effect of varying concentrations of NSC-641296 (A), NSC-668394 (B), NSC-158011 (C) and NSC-323241 (D) chemical compounds on the catalytic activity of HcPMT1 + HcPMT2 combination (solid line) or HcPMT1 only (dashed line) proteins in the methyltransferase assay. The data shown represent means of three independent experiments with standard error bars.

percentage of sluggishly motile worms was at least 3-fold significantly ($P < 0.001$) higher than that of very motile worms. Additionally, at 22 μM of NSC-641296, the percentage of immotile worms was 4-fold significantly ($P < 0.001$) higher than the

Table 2
Chemical compounds' IC_{50} values for inhibition of catalytic activity of HcPMT1 only and HcPMT1 + HcPMT2 combination.

Compound	HcPMT1 Activity inhibition IC_{50} (μM)	HcPMT1 + HcPMT2 Activity inhibition IC_{50} (μM)
NSC-641296	8.3 ± 1.1	5.1 ± 1.8
NSC-668394	5.9 ± 0.9	2.8 ± 0.6
NSC-158011	ND*	ND*
NSC-323241	ND*	ND*

ND* = not derivable because of low inhibitory activity.

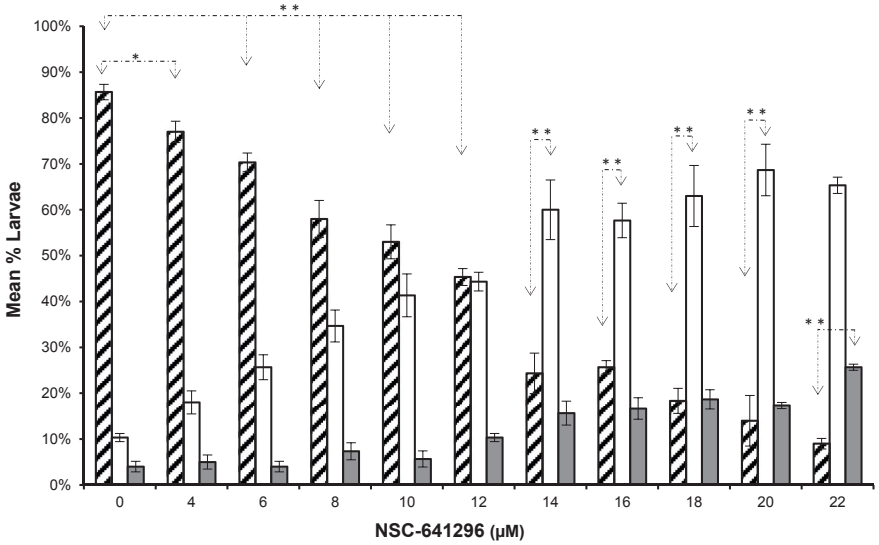

Fig. 5. Analysis of the effect of varying concentrations of chemical compound NSC-641296 on the motile activity of *H. contortus* third-stage larvae after 36 h of culture in supplemented RPMI-1640 medium. For each culture, 50 larvae were counted and the percentage of very motile larvae (hatched columns), sluggishly motile larvae (white columns) and non-motile larvae (gray columns) determined. The data shown represent means of three independent experiments with standard error bars. Arrow-heads indicate the column data that is different with the level of significance denoted by asterisks (*P < 0.05; * *P < 0.001).

percentage of very motile worms. Overall, the number of actively motile worms decreased significantly ($P < 0.001$), while the number of sluggishly motile and non-motile larvae increased significantly ($P < 0.001$) in a dose-dependent format (Fig. 5), indicating that the compound had anthelmintic activity against *H. contortus* larvae. Likewise, in cultures of adult worms treated with compound NSC-641296, after 36 h there was a significant ($P < 0.05$) reduction in the percentage of motile worms and a corresponding significant ($P < 0.001$) increase in the number of immotile worms, starting at 4 μM of NSC-641296 in medium (Fig. 6). At 8 μM and above, the percentage of immotile worms was at least 2-fold significantly ($P < 0.001$) higher than that of motile worms (Fig. 6). Overall, there was a significant ($P < 0.001$) compound dose-dependent reduction in the percentage of motile worms, with a corresponding significant ($P < 0.001$) increase in the percentage of non-motile worms (Fig. 6) suggesting that the compound had anthelmintic activity against the adult worms as well. Coincidentally, the number of motile worms in the NSC-641296-treated cultures was observed to decrease by half at a concentration of NSC-641296 (8 μM) that depicted the highest inhibitory effect against the catalytic activity of HcPMT1 and HcPMT2. By non-linear regression analysis, we derived the IC_{50} of NSC-641296 to be about 7 ± 1.8 μM and 15 ± 2.9 μM against adult worms and larval worms, respectively. Analysis of the toxic concentrations of compound NSC-641296 in mammalian monocytic cells showed that its cytotoxic IC_{50} was 29 ± 2.1 μM, which was 4-fold and 2-fold higher than its anthelmintic IC_{50} against adult worms and larvae, respectively.

Compound NSC-668394, that also significantly inhibited the catalytic activity of HcPMT1 and HcPMT2, did not show any

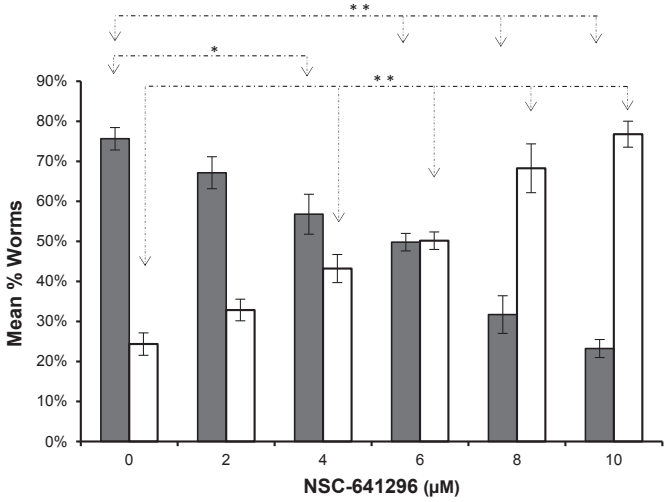

Fig. 6. Analysis of the effect of varying concentrations of chemical compound NSC-641296 on the motile activity of adult female *H. contortus* worms after 36 h of culture in supplemented RPMI-1640 medium. For each culture, 50 worms were counted and the percentage of motile worms (dark columns) and non-motile worms (white columns) determined. The data shown represent means of three independent experiments with standard error bars. Arrow-heads indicate the column data that is different with the level of significance denoted by asterisks (*P < 0.05; * *P < 0.001).

significant anthelmintic effect against both larvae and adult *H. contortus* (data not shown) even at a concentration (60 μM) that was 12-fold higher than the concentration (5 μM) at which it was

found to exhibit the highest inhibitory effect on HcPMT1 and HcPMT2 activity. Compounds NSC-158011 and NSC-323241 that had comparatively lower inhibitory effect against HcPMT1 and HcPMT2 activity also did not show any anthelmintic effect against either the adult worms or the larvae at all the concentrations tested up to a maximum of 80 μM (data not shown).

4. Discussion

Small ruminants, despite their resilience are known to be highly susceptible to the stomach worm, *H. contortus* (Soulsby, 1982). The control of *H. contortus* relies heavily on the use of anthelmintic drugs, but multi-drug resistant populations of *H. contortus* are now widespread and pose a major threat to the small ruminant industry worldwide (Kaplan, 2004; Sargison et al., 2007). Thus, there is urgent need to identify, characterize and validate novel molecular drug targets as well as to identify their specific chemical inhibitors in *H. contortus* for developing a new generation of effective drugs against *H. contortus* and other helminths.

The phosphobase methylation pathway for the biosynthesis of phosphatidylcholine is unique to nematodes, plants and some apicomplexan protozoa (McCarter, 2004; Mitreva et al., 2005), making it an ideal molecular target for developing new anthelmintic agents. Herein, we screened chemical compounds with the goal of identifying those with inhibitory activity against enzymes catalyzing the phosphobase methylation step in *H. contortus*.

To screen the chemical compounds, we utilized the commercially available Methyltransferase HT Activity Assay kit (ENZO) in which we used natively purified HcPMT1 and HcPMT2 recombinant proteins as the methyltransferase enzymes. When HcPMT2 was used alone as the enzyme, there was no notable catalytic activity, while HcPMT1 alone depicted significant catalytic activity that was augmented by combining HcPMT1 and HcPMT2 as enzymes in the assay. Unlike *P. falciparum* which possesses a single methyltransferase that catalyzes all three steps of methylation of phosphoethanolamine (Pessi et al., 2004), *H. contortus* possesses two enzymes, HcPMT1 and HcPMT2, that sequentially methylate phosphoethanolamine (PE) to phosphocholine (Lee et al., 2011). HcPMT1 possesses an N-terminal methyltransferase domain that catalyzes the first step of methylating PE to phosphomonomethylethanolamine (PMME). On the other hand, HcPMT2 possesses the C-terminal domain that functions to catalyze the subsequent two methylation steps of converting PMME to phosphodimethylethanolamine (PDME) and methylating PDME to phosphocholine (Brendza et al., 2007; Lee and Jez, 2013). This is consistent with the observation that HcPMT2 could not catalyze the methylation of PE while HcPMT1 did, leading to the generation of PMME, a substrate for HcPMT2 resulting in increased detectable fluorescence due to increased generation of *S*-adenosylhomocysteine. This in-concert catalytic activity of HcPMT1 and HcPMT2 for synthesis of phosphocholine provides two potential drug targets in the phosphobase methylation pathway of *H. contortus*. Interestingly, we observed that HcPMT1 and HcPMT2 expression levels in *H. contortus* are similar, consistent with their in-concert catalytic activity.

Among the chemical compounds tested, NSC-641296 and NSC-668394 that significantly inhibited the activity of HcPMT1 as a sole enzyme also more effectively inhibited the combined activity of HcPMT1 and HcPMT2 than compounds NSC-158011 and NSC-323241 that did not inhibit HcPMT1 as a sole enzyme. Interestingly, in previous studies, both NSC-158011 and NSC-323241 were found to have significant inhibitory effect against the catalytic activity of the *P. falciparum* PMT comparable to the inhibitory activities of NSC-641296 and NSC-668394 (Bobenchik et al., 2013). Unlike *H. contortus* which has two PMT enzymes, *P. falciparum* possesses a single multifunctional PMT enzyme that contains a single methyltransferase domain responsible for all three methylation steps (Lee and Jez, 2013). The *P. falciparum* enzyme is more closely related to *H. contortus* HcPMT2 in structure of the catalytic domain (Lee and Jez, 2014). This is consistent with our observation that compounds NSC-158011 and NSC-323241 that have been shown to significantly inhibit the activity of the *P. falciparum* PMT had no inhibitory effect against *H. contortus* HcPMT1 which initiates the methylation of PE.

When tested for anthelmintic activity against *H. contortus* adult and larval worms, only compound NSC-641296 had potent antiparasitic activity. It was, however, surprising that NSC-668394, which had inhibitory effect against HcPMT1 and HcPMT2 enzymes comparable to that of NSC-641296, did not show any anthelmintic activity against *H. contortus* adult or larval worms. This can possibly be attributed to differences in uptake of the compounds by the worm cells because the chemical structures of the two compounds are significantly different (Fig. 7). Despite the notable anthelmintic effect of NSC-641296 against third-stage larvae and adult *H. contortus*, with IC_{50} values of 15 ± 2.9 μM and 7 ± 2.9 μM, respectively, when compared to its cytotoxic IC_{50} (29 ± 2.1 μM) in a mammalian cell line, it is evident that this compound has low selectivity which would impact its usefulness. Therefore, although the targeted pathway is nematode-specific, this fact alone cannot be used as a strong argument for the ability to readily develop nematode-specific drugs. Nevertheless, chemical structure modifications to optimize compound solubility, stability, cellular uptake and inhibitory activity can potentially enhance the anthelmintic activity, while reducing the level of toxicity to mammalian cells for NSC-641296 (Fig. 7A) and possibly that of NSC-668394 (Fig. 7B). NSC-158011 and NSC-323241 that have both been reported to have significant antiparasitic activity against *P. falciparum* (Bobenchik et al., 2013) did not show any anthelmintic activity against *H. contortus*, consistent with their modest inhibitory effect on the combined catalytic activity of HcPMT1 and HcPMT2.

NSC-641296 depicted more potent activity against adult *H. contortus* worms than against the larvae. Unlike adult *H. contortus* worms, third-stage larvae are protected by a sheath that prevents them from feeding (Cantacessi et al., 2012) and this may make them accumulate less of the chemical inhibitor than the adult worms would. Despite the presence of the alternative Kennedy pathway that utilizes choline as a precursor for the

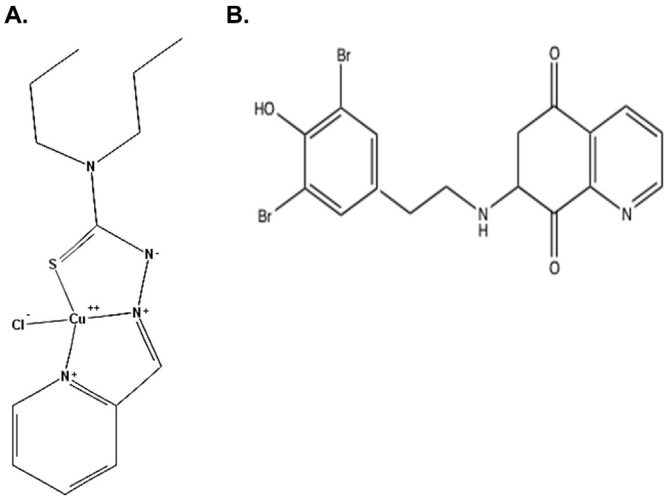

Fig. 7. Illustrations of the chemical structures of compounds 641296 (**A**) and 668394 (**B**).

biosynthesis of phosphatidylcholine in nematodes, our findings here indicate that inhibition of the phosphobase pathway by chemical compounds cannot be compensated for by the alternative Kennedy pathway. With the prevailing populations of *H. contortus* that are resistant to multiple anthelmintics, the identification of chemical compounds that inhibit unique essential biosynthetic pathways in *H. contortus* provides impetus to the development of a new generation of efficacious anthelmintic drugs.

In parasitic nematodes, resistance to some anthelmintics has been associated with mutations in genes encoding the drugs' molecular targets (Kwa et al., 1994; Ashraf et al., 2015). Inhibitors of phosphoethanolamine methyltransferases have been shown to bind the catalytic site of the enzymes to block the substrate/co-substrate binding (Lee et al., 2012). The catalytic domains of phosphoethanolamine methyltransferases are conserved (Lee and Jez, 2013) and mutations in the domains lead to loss of function (Reynolds et al., 2008) which can be detrimental to the survival of the parasite. This may reduce the likelihood of resistance to drugs that inhibit the phosphobase pathway arising through mutations in the genes encoding the methyltransferases.

Acknowledgments

This study was funded in part by the USDA/NIFA/AFRI grant number 2014-67016-21570 to WHW. Competing interests: None.

References

Ashraf, S., Mani, T., Beech, R., Prichard, R., 2015. Macrocyclic lactones and their relationship to the SNPs related to benzimidazole resistance. Mol. Biochem. Parasitol. 201, 128–134.

Bobenchik, A.M., Witola, W.H., Augagneur, Y., Nic Lochlainn, L., Garg, A., Pachikara, N., Choi, J.Y., Zhao, Y.O., Usmani-Brown, S., Lee, A., Adjalley, S.H., Samanta, S., Fidock, D.A., Voelker, D.R., Fikrig, E., Ben Mamoun, C., 2013. *Plasmodium falciparum* phosphoethanolamine methyltransferase is essential for malaria transmission. Proc. Natl. Acad. Sci. U. S. A. 110, 18262–18267.

Brendza, K.M., Haakenson, W., Cahoon, R.E., Hicks, L.M., Palavalli, L.H., Chiapelli, B.J., McLaird, M., McCarter, J.P., Williams, D.J., Hresko, M.C., Jez, J.M., 2007. Phosphoethanolamine N-methyltransferase (PMT-1) catalyses the first reaction of a new pathway for phosphocholine biosynthesis in *Caenorhabditis elegans*. Biochem. J. 404, 439–448.

Cantacessi, C., Campbell, B.E., Gasser, R.B., 2012. Key strongylid nematodes of animals - impact of next-generation transcriptomics on systems biology and biotechnology. Biotechnol. Adv. 30, 469–488.

Elabbadi, N., Ancelin, M.L., Vial, H.J., 1997. Phospholipid metabolism of serine in *Plasmodium*-infected erythrocytes involves phosphatidylserine and direct serine decarboxylation. Biochem. J. 324, 435–445.

Gilleard, J.S., 2006. Understanding anthelmintic resistance: the need for genomics and genetics. Int. J. Parasitol. 36, 1227–1239.

Kaplan, R.M., 2004. Drug resistance in nematodes of veterinary importance: a status report. Trends Parasitol. 20, 477–481.

Kent, C., 1995. Eukaryotic phospholipid biosynthesis. Annu. Rev. Biochem. 64, 315–343.

Kwa, M.S., Veenstra, J.G., Roos, M.H., 1994. Benzimidazole resistance in *Haemonchus contortus* is correlated with a conserved mutation at amino acid 200 in b-tubulin isotype 1. Mol. Biochem. Parasitol. 63, 299–303.

Lee, S.G., Alpert, T.D., Jez, J.M., 2012. Crystal structure of phosphoethanolamine methyltransferase from *Plasmodium falciparum* in complex with amodiaquine. Bioorg. Med. Chem. Lett. 22, 4990–4993.

Lee, S.G., Haakenson, W., McCarter, J.P., Williams, D.J., Hresko, M.C., Jez, J.M., 2011. Thermodynamic evaluation of ligand binding in the plant-like phosphoethanolamine methyltransferases of the parasitic nematode *Haemonchus contortus*. J. Biol. Chem. 286, 38060–38068.

Lee, S.G., Jez, J.M., 2013. Evolution of structure and mechanistic divergence in Di-domain methyltransferase from nematode phosphocholine biosynthesis. Structure 21, 1778–1787.

Lee, S.G., Jez, J.M., 2014. Nematode phospholipid metabolism: an example of closing the genome-structure-function circle. Trends Parasitol. 30, 241–250.

McCarter, J.P., 2004. Genomic filtering: an approach to discovering antiparasitics. Trends Parasitol. 20, 462–468.

Mitreva, M., Appleton, J., McCarter, J.P., Jasmer, D.P., 2005. Expressed sequence tags from life cycle stages of *Trichinella spiralis*: application to biology and parasite control. Vet. Parasitol. 132, 13–17.

Mortensen, L.L., Williamson, L.H., Terrill, T.H., Kircher, R.A., Larsen, M., Kaplan, R.M., 2003. Evaluation of prevalence and clinical implications of anthelmintic resistance in gastrointestinal nematodes in goats. J. Am. Vet. Med. Assoc. 23, 495–500.

Nikolaou, S., Gasser, R.B., 2006. Prospects for exploring molecular developmental processes in *Haemonchus contortus*. Int. J. Parasitol. 36, 859–868.

Palavalli, L.H., Brendza, K.M., Haakenson, W., Cahoon, R.E., McLaird, M., Hicks, L.M., McCarter, J.P., Williams, D.J., Hresko, M.C., Jez, J.M., 2006. Defining the role of phosphomethylethanolamine N-methyltransferase from *Caenorhabditis elegans* in phosphocholine biosynthesis by biochemical and kinetic analysis. Biochemistry 45, 6056–6065.

Pena, M.T., Fonetenot, M.E., Miller, J.E., Gillespie, A., Larsen, M., 2002. Evaluation of the efficacy of Duddingtonia flagrans in reducing infective larvae of *Haemonchus contortus* in faeces of sheep. Vet. Parasitol. 103, 259–265.

Pessi, G., Choi, J.Y., Reynolds, J.M., Voelker, D.R., Mamoun, C.B., 2005. In vivo evidence for the specificity of *Plasmodium falciparum* phosphoethanolamine methyltransferase and its coupling to the Kennedy pathway. J. Biol. Chem. 280, 12461–12466.

Pessi, G., Kociubinski, G., Mamoun, C.B., 2004. A pathway for phosphatidylcholine biosynthesis in *Plasmodium falciparum* involving phosphoethanolamine methylation. Proc. Natl. Acad. Sci. U. S. A. 101, 6206–6211.

Rhoads, M.L., Fetterer, R.H., 1995. Developmentally regulated secretion of cathepsin L-like cysteine proteases by *Haemonchus contortus*. J. Parasitol. 81, 505–512.

Reynolds, J.M., Takebe, S., Choi, J.Y., El Bissati, K., Witola, W.H., Bobenchik, A.M., Hoch, J.C., Voelker, D.R., Mamoun, C.B., 2008. Biochemical and genetic analysis of the phosphoethanolamine methyltransferase of the human malaria parasite *Plasmodium falciparum*. J. Biol. Chem. 283, 7894–7900.

Sargison, N.D., Jackson, F., Bartley, D.J., Wilson, D.J., Stenhouse, L.J., Penny, C.D., 2007. Observations on the emergence of multiple anthelmintic resistance in sheep flocks in the south-east of Scotland. Vet. Parasitol 145, 65–76.

Soulsby, E.J.L., 1982. Helminths, Arthropods and Protozoa of Domesticated Animals, seventh ed. Baillière Tindall, London, UK.

Vial, H.J., Ancelin, M.L., 1998. In: Sherman, I.W. (Ed.), Malaria Lipids. Malaria Parasite Biology, Pathogenesis and Protection. Am. Soc. Microbiol. Press, Washington, pp. 159–175.

Witola, W.H., El Bissati, K., Pessi, G., Xie, C., Roepe, P.D., Mamoun, C.B., 2008. Disruption of the *Plasmodium falciparum* PfPMT gene results in a complete loss of phosphatidylcholine biosynthesis via the serine-decarboxylase-phosphoethanolamine-methyltransferase pathway and severe growth and survival defects. J. Biol. Chem. 283, 27636–27643.

Zarlenga, D.S., Stringfellow, F., Nobary, M., Lichtenfels, J.R., 1994. Cloning and characterization of ribosomal RNA genes from three species of Haemonchus (Nematoda: Trichostrongyloidea) and identification of PCR primers for rapid differentiation. Exp. Parasitol. 78, 28–36.

Ziegler-Heitbrock, H.W., Thiel, E., Fütterer, A., Herzog, V., Wirtz, A., Riethmüller, G., 1988. Establishment of a human cell line (Mono Mac 6) with characteristics of mature monocytes. Int. J. Cancer 41, 456–461.