Parasitology Research (2020) 119:165–175
https://doi.org/10.1007/s00436-019-06492-7

HELMINTHOLOGY - ORIGINAL PAPER

In vitro apoptotic effect on human lymphatic filarial parasite by piperidine derivatives and thymidine reversal study

Priyanka S. Bhoj[1] · Sahitya Rao[1] · Sandeep P. Bahekar[2] · Nikita R. Agrawal[2] · Namdev S. Togre[1] · Richa Sharma[1] · Kalyan Goswami[1] · Hemant S. Chandak[2] · Mandakini B. Patil[3]

Received: 8 September 2018 / Accepted: 30 September 2019 / Published online: 5 December 2019

Abstract

A novel library of synthetic piperidine derivatives was used to screen against human lymphatic filarial parasite *Brugia malayi*. Piperidine has earlier been reported to have effect against parasites including rodent filarial nematodes. Compounds with hydroxyl substitutions (4Q and 4H) showed marked antifilarial effect. Molecular docking of 4H derivative showed more favorable thermodynamic parameters against thymidylate synthase of *B. malayi* than human counterpart. A wide difference between IC_{50} and LD_{50} ensured the therapeutic safety of the candidates against the filarial parasites. Addition of thymidine to the treatment regimen led to a significant reversal of antifilarial effect of 4H that confirmed inhibition of thymidylate synthase as pharmacological rationale. Apoptosis induced in the parasite as a consequence of probable inhibition of thymidylate synthase was studied by acridine orange/ethidium bromide fluorescent staining and poly (ADP-ribose) polymerase activity inhibition. Involvement of mitochondria was confirmed by decreased 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) conversion and increased cytosolic cytochrome c level in 4H treated microfilariae, compared with the untreated microfilariae. Moreover, Michael adduct of chalcone targeting dihydrofolate reductase and piperidine targeting thymidylate synthase demonstrated synergistic effect on the parasite, indicating the importance of inhibition of DNA synthesis by combined effect. In conclusion, piperidine derivatives with hydroxyl substitution have a great therapeutic potential with an apoptotic rationale involving mitochondrial pathway, due to possible inhibition of parasitic thymidylate synthase.

Keywords Piperidine · Thymidylate synthase · Apoptosis · Folate metabolism · *Brugia malayi*

Introduction

Lymphatic filariasis is a profoundly disabling and debilitating vector-borne disease affecting nearly 120 million people in tropical and subtropical countries worldwide. Diethylcarbamazine (DEC) has been the cornerstone of the mass drug administration (MDA) scheme initiated by the WHO under Global programme for elimination of lymphatic filariasis (GPELF) (World Health Organization 2017). However, the data on the mechanistic detail of this piperazine derivative is lacking. Apparently, this drug largely depends on host macrophages for its possible action (Maizels and Denham 1992).

Nomenclature of piperazine is etymologically linked to piperidine owing to its structural resemblance having a common structural motif, piperine derived from black pepper. Piperazines are solely synthetic compounds used in antihelminthic drugs like DEC. On the other hand, piperidine compounds are more versatile heterocyclic amines that are either synthesized or obtained naturally from black pepper, *Psilocaulon absimile* N.E.Br (Aizoaceae) and *Petrosimonia monandra* (Rimington 1934; Mahalakshmi and Balachandran 2014). Earlier, we have successfully targeted folate metabolism in *Brugia malayi* with resultant

Priyanka S. Bhoj and Sahitya Rao contributed equally to this work.

Section Editor: Ramaswamy Kalyanasundaram

✉ Kalyan Goswami
goswamikln@gmail.com

✉ Hemant S. Chandak
chemants@gmail.com

[1] Department of Biochemistry, Mahatma Gandhi Institute of Medical Sciences, Sevagram, Wardha 442 102, India

[2] Department of Chemistry, G. S. Science, Arts and Commerce College, Khamgaon 444303, India

[3] University Department of Biochemistry, RTM Nagpur University, Nagpur 440033, India

apoptosis, which unequivocally indicated the dependence of this parasite for folate-mediated DNA synthesis (Sharma et al. 2013). As DNA synthesis is essential for cell proliferation, inhibition of this vital metabolism is a popular rationale against wide range of microbial organisms including parasites. Although DEC is devoid of direct action against filarial parasite, it shows weak antifolate activity (Gupta and Srivastava 2005). In this context, antifolate compounds like pyrimethamine and di-amino pyrimidine provide a classic prototype (Falco et al. 1951; Nelson and Rosowsky 2001). Owing to the critical role of the thymidine, it is considered to be the signature molecule in the DNA synthesis. Therefore, thymidylate synthase (TS) enzyme of the DNA synthesis pathway might be contemplated as an effective target. The so-called thymine-less death has been exploited for anticancer therapeutics (Houghton et al. 1997); however, such stratagem remained relatively unexplored in antimicrobial drug development. Interestingly, piperidine analogs of 5-fluoro-2'-deoxyuridine have been reported to inhibit TS (Fries et al. 1995). Pyrimidine derivatives substituted with piperidine have been shown to be endowed with antileishmanial potential (Sharma et al. 2014). Also, it has been shown to have filaricidal efficacy against rodent filarial nematodes *Acanthocheilonema viteae* and *Litomosoides carinii* in in vivo (Agarwal et al. 2011).

Hence, herein we explored the possible inhibitory effect of a novel library of synthetic piperidine derivatives on human lymphatic filarial pathogen *Brugia malayi* and its therapeutic rationale.

Materials and methods

Materials

The reagents, chemicals, and other apparatus were procured from commercial sources (Himedia Laboratories Pvt. Ltd, SD Fine Chemicals, Sisco Research Laboratories, etc). The library of synthetic piperidine compounds (Table 1) was synthesized and characterized as described earlier (Agrawal et al. 2015).

Collection of *B. malayi* microfilariae

The infection of the filarial parasite (*B. malayi*) was established and maintained in jirds (*Meriones unguiculatus*), mastomys (*Mastomys natelansis*), and mosquitoes (*Aedes aegypti*) using standard methods (Ash and Riley 1970; Sanger et al. 1981) at the institutional animal house. Microfilariae (Mf) were obtained by lavage of the peritoneal cavities of infected jirds, washed with RPMI-1640 medium twice at 1000 rpm for 5 min, plated on sterile plastic Petri dishes, and incubated at 37 °C for 1 h to remove jird's peritoneal exudate cells. The Mf were then collected from the Petri dishes, washed with RPMI-1640 medium and used for in vitro experiments.

In vitro screening of antifilarial activity of the proposed drugs

The library of synthetic piperidine compounds was screened to select effective compound(s). For initial screening, a stock of 50 mM was prepared in dimethyl sulfoxide (DMSO). Further dilutions were made to obtain the desired final concentration in the range of 0.5 to 500 μM in 900 μL of 0.9% normal saline. A simultaneous control was taken with DMSO (1%) as a vehicle control without the drug solution in 900 μL saline. Approximately, 100 Mf in 100 μL saline were added into the above vials and incubated for 30 min at 37 °C in a shaking incubator at 150 rpm. After 30-min incubation, the Mf were collected by centrifugation, washed with fresh RPMI medium, and transferred to 24 well culture plates making the final volume 1 mL with RPMI medium. The plates were incubated at 37 °C for 48 h in 5% CO_2. Mf motility was assessed by an inverted microscope after 48 h. The observations were recorded as the percentage of non-motile Mf in each well. Each experiment was repeated thrice to check for reproducibility (Bahekar et al. 2016). The concentrations at which 50% and 100% loss of Mf motility were observed and have been denoted as IC_{50} and IC_{100} concentration, respectively.

Protein modeling

The amino acid sequence of TS protein of *B. malayi* (accession number: XM_001898103.1) and human (accession number: 4 UP1_A) was retrieved from the National Center for Biotechnology Information (NCBI) database. Three-dimensional structure of human TS is available in the Protein Data Bank. As the three-dimensional structure of the corresponding *B. malayi* protein is not available in the Protein Data Bank, the 3-D model was developed.

The 3-D protein structure was modeled by uploading the FASTA format of protein sequence in Phyre2 server. PROCHECK program and ProSA-web were employed for validation of predicted protein structures. The visualization of models was performed using PyMOL software.

Molecular docking

For molecular docking, the molecular structures of ligands (i.e., 4Q and 4H) were drawn using online PRODRG server. Molecular docking was performed using AutoDock tool 4.2 version as per the user guide. Briefly, the PDB file of protein and ligands was added with hydrogen and saved in PDBQT format. Calculation of affinity maps was done by AutoGrid. For calculation of affinity maps at the catalytic site of BmTS,

Table 1 Structures, molecular weights, and IUPAC names of piperidine compounds screened along with their antifilarial activity

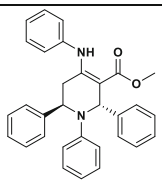
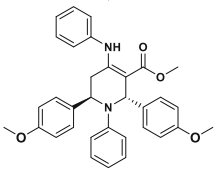
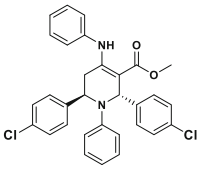
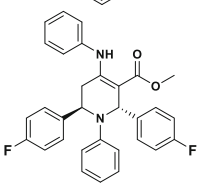
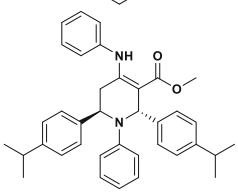
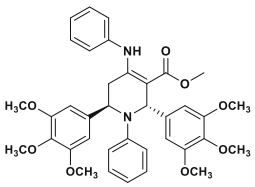
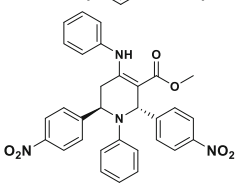
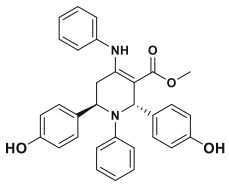
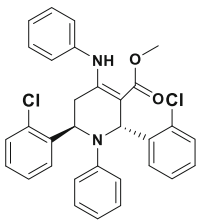
Mol ID	Structure	IUPAC Name	Activity*
4A		Methyl (2S,6R)-1,2,6-triphenyl-4-(phenylamino)-1,2,5,6-tetrahydropyridine-3-carboxylate	22.25 ± 2.62
4B		Methyl (2S,6R)-2,6-bis(4-methoxyphenyl)-1-phenyl-4-(phenylamino)-1,2,5,6-tetrahydropyridine-3-carboxylate	19 ± 3.36
4C		Methyl (2S,6R)-2,6-bis(4-chlorophenyl)-1-phenyl-4-(phenylamino)-1,2,5,6-tetrahydropyridine-3-carboxylate	35.5 ± 5.44
4D		Methyl (2S,6R)-2,6-bis(4-fluorophenyl)-1-phenyl-4-(phenylamino)-1,2,5,6-tetrahydropyridine-3-carboxylate	34 ± 5.35
4E		Methyl (2S,6R)-2,6-bis(4-isopropylphenyl)-1-phenyl-4-(phenylamino)-1,2,5,6-tetrahydropyridine-3-carboxylate	39 ± 7.58
4F		Methyl (2S,6R)-1-phenyl-4-(phenylamino)-2,6-bis(3,4,5-trimethoxyphenyl)-1,2,5,6-tetrahydropyridine-3-carboxylate	18 ± 2.44
4G		Methyl (2S,6R)-2,6-bis(4-nitrophenyl)-1-phenyl-4-(phenylamino)-1,2,5,6-tetrahydropyridine-3-carboxylate	20 ± 5.88
4H		Methyl (2S,6R)-2,6-bis(4-hydroxyphenyl)-1-phenyl-4-(phenylamino)-1,2,5,6-tetrahydropyridine-3-carboxylate	100 ± 0.0
4I		Methyl (2R,6R)-2,6-bis(2-chlorophenyl)-1-phenyl-4-(phenylamino)-1,2,5,6-tetrahydropyridine-3-carboxylate	36.75 ± 4.27

Table 1 (continued)

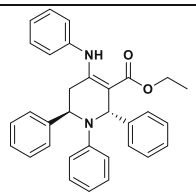
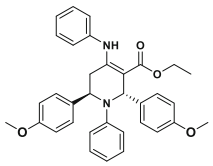
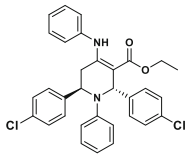
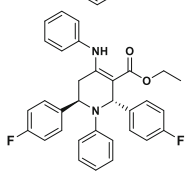
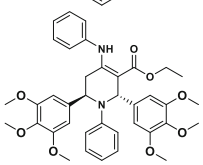
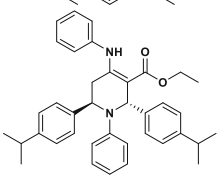
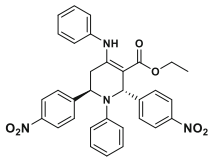
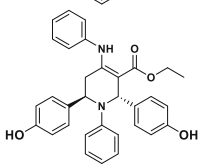
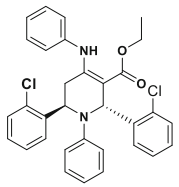
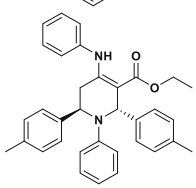
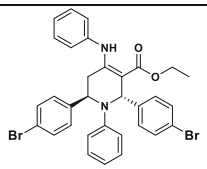
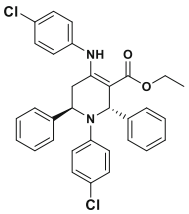
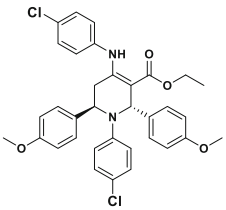
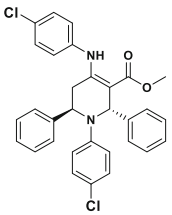
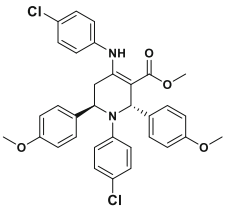
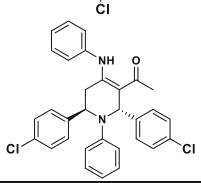
4J		Ethyl (2S,6R)-1,2,6-triphenyl-4-(phenylamino)-1,2,5,6-tetrahydropyridine-3-carboxylate	47.25 ± 5.12
4K		Ethyl (2S,6R)-2,6-bis(4-methoxyphenyl)-1-phenyl-4-(phenylamino)-1,2,5,6-tetrahydropyridine-3-carboxylate	29 ± 6.055
4L		Ethyl (2S,6R)-2,6-bis(4-chlorophenyl)-1-phenyl-4-(phenylamino)-1,2,5,6-tetrahydropyridine-3-carboxylate	32 ± 6.21
4M		Ethyl (2S,6R)-2,6-bis(4-fluorophenyl)-1-phenyl-4-(phenylamino)-1,2,5,6-tetrahydropyridine-3-carboxylate	31.25 ± 4.78
4O		Ethyl (2S,6R)-1-phenyl-4-(phenylamino)-2,6-bis(3,4,5-trimethoxyphenyl)-1,2,5,6-tetrahydropyridine-3-carboxylate	33.5 ± 4.43
4N		Ethyl (2S,6R)-2,6-bis(4-isopropylphenyl)-1-phenyl-4-(phenylamino)-1,2,5,6-tetrahydropyridine-3-carboxylate	29.25 ± 4.42
4P		Ethyl (2S,6R)-2,6-bis(4-nitrophenyl)-1-phenyl-4-(phenylamino)-1,2,5,6-tetrahydropyridine-3-carboxylate	30 ± 3.9
4Q		Ethyl (2S,6R)-2,6-bis(4-hydroxyphenyl)-1-phenyl-4-(phenylamino)-1,2,5,6-tetrahydropyridine-3-carboxylate	100 ± 0.0
4R		Ethyl (2R,6R)-2,6-bis(2-chlorophenyl)-1-phenyl-4-(phenylamino)-1,2,5,6-tetrahydropyridine-3-carboxylate	16.25 ± 3.09
4S		Ethyl (2S,6R)-1-phenyl-4-(phenylamino)-2,6-di-p-tolyl-1,2,5,6-tetrahydropyridine-3-carboxylate	58.5 ± 4.43

Table 1 (continued)

4T		Ethyl (2S,6R)-2,6-bis(4-bromophenyl)-1-phenyl-4-(phenylamino)-1,2,5,6-tetrahydropyridine-3-carboxylate	48.75 ± 4.57
4U		Ethyl (2S,6R)-1-(4-chlorophenyl)-4-((4-chlorophenyl)amino)-2,6-diphenyl-1,2,5,6-tetrahydropyridine-3-carboxylate	20.5 ± 3.87
4V		Ethyl (2S,6R)-1-(4-chlorophenyl)-4-((4-chlorophenyl)amino)-2,6-bis(4-methoxyphenyl)-1,2,5,6-tetrahydropyridine-3-carboxylate	38.25 ± 7.13
4W		Methyl (2S,6R)-1-(4-chlorophenyl)-4-((4-chlorophenyl)amino)-2,6-diphenyl-1,2,5,6-tetrahydropyridine-3-carboxylate	19 ± 2.16
4X		Methyl (2S,6R)-1-(4-chlorophenyl)-4-((4-chlorophenyl)amino)-2,6-bis(4-methoxyphenyl)-1,2,5,6-tetrahydropyridine-3-carboxylate	35.25 ± 4.99
4Y		1-((2S,6R)-2,6-bis(4-chlorophenyl)-1-phenyl-4-(phenylamino)-1,2,5,6-tetrahydropyridin-3-yl)ethan-1-one	14.25 ± 6.701

*Results are expressed as mean ± SD of % loss of motility of Mf at 500-μM concentration after 48 h

grid center was fixed at $X = -141.077$, $Y = -11.292$, $Z = 222.425$, and spacing 0.375. For human TS, grid centre was fixed at $X = 10.151$, $Y = 40.101$, $Z = 24.936$, and spacing 0.375. Docking simulation was executed using the Lamarckian genetic algorithm. Consequently, the results with the best free energy of binding (ΔG_b) and inhibition constant (K_i) were selected.

Evaluation of the in vitro cytotoxicity of effective compounds

Whole blood samples were obtained from healthy human volunteers after receiving their informed consent. Venous blood was overlaid carefully on histopaque-1077 (Sigma-Aldrich, USA) in 1:1 ratio taken in tubes and centrifuged at room temperature at 400*g* for 30 min. The upper layer of plasma was discarded, and opaque layer of about 0.5 cm containing PBMCs was collected. This layer was re-suspended in RPMI-1640 medium and centrifuged at 250*g* for 10 min, and the cell pellet was collected. The washing procedure was repeated twice, and the cell pellet was re-suspended in RPMI-1640 medium supplemented with 10% fetal bovine serum.

Cytotoxicity was evaluated against human PBMCs (1×10^5 PBMCs/mL) by trypan-blue exclusion assay (Bhoj et al. 2018). The dose at which 50% cytotoxicity was observed has been denoted as LD_{50} concentration (Mandvikar et al. 2016).

Reversal of the activity of piperidine compounds by thymidine

In an attempt to probe the mechanism of action of these compounds, reversal studies using thymidine (product of TS enzyme) were carried out. As per the previous experiment for in vitro effect of the drug on the parasite, around 100 Mf were pre-incubated with 100 μM of freshly prepared thymidine solution for 1 h (pre-optimized condition). Further, thymidine pre-incubated Mf were washed with saline and then treated with the test compound at IC_{90} (a dose at which 90% loss of Mf motility was achieved). After drug treatment for 30 min, Mf were centrifuged and transferred to the 24 well culture plates containing RPMI-1640 medium for incubation maintaining 5% CO_2 at 37 °C for 48 h. After incubation, the percentage reduction in loss of Mf motility was recorded.

Ethidium bromide-acridine orange staining

Fluorescent staining with ethidium bromide-acridine orange (EB/AO) was carried out as per the standard protocol (Singh et al. 2012). The dye mix comprised EB and AO 100 μg/mL each, respectively in phosphate-buffered saline (pH 7.2). Mf treated with the test compound or staurosporine (20 μM; as a positive control for the induction of apoptosis) along with negative control (only DMSO treated Mf) were washed and re-suspended in 25 μL cold PBS, followed by the addition of 5-μL EB/AO dye mix. Stained Mf were viewed under an epifluorescence microscope (Nikon LABOPHOT) with the excitation filter set at 480/30 nm and barrier filter at 535/40 nm. Tests were carried out in triplicate, counting a minimum of 10 Mf in each observation for detection of differential staining.

Poly (ADP-ribose) polymerase activity assay

Poly (ADP-ribose) polymerase (PARP) activity in *B. malayi* Mf was determined using a commercial kit (R & D Systems Inc, Minneapolis, MD) according to the manufacturer's instructions. Briefly, 100-μL aliquots of suspension (containing about 10,000 Mf), treated with the drug, were lysed with radioimmunoprecipitation assay buffer (RIPA buffer; Sigma-Aldrich, Bangalore) for 1 h in the presence of protease inhibitors. Lysate (20 μg) was added to each well in 96-well plates pre-coated with histone. PARP activity was determined from the incorporation of biotinylated poly (ADP-ribose) onto immobilized histone, which was measured by the addition of streptavidin-conjugated horseradish peroxidase and a suitable chromogenic substrate to the incubation mixture. A standard curve for PARP enzymatic activity (A450 versus PARP units) was initially plotted using 0.01, 0.05, 0.1, 0.5, and 1 unit of enzyme per well. The absorbance obtained with each test sample (Mf lysate) was extrapolated on the standard curve to obtain the corresponding PARP activity. The percentage inhibition in enzymatic activity in the test sample was accordingly calculated. The experiment was carried out in triplicate (Singh et al. 2012).

MTT assay

Treated and untreated Mf incubated for 48 h were transferred to 100 μL PBS (0.05 mM, pH 7.2) containing 0.5 mg/mL of 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT; Sigma-Aldrich, Bangalore) and then incubated for 2 h at 37 °C in the dark. The Mf were pelleted and washed once with PBS. To the Mf pellet, 100 μL of DMSO was added and incubated for 1 h to dissolve the dark blue crystals of formazan. Quantification was performed at 595 nm (Rao et al. 1991).

Cytochrome c estimation

Both treated and untreated Mf were lysed with RIPA buffer (Himedia Laboratories Pvt Ltd, Mumbai) for 1 h in the presence of protease inhibitors. The Mf lysates were centrifuged at 1000*g* for 10 min at 4 °C to remove the cell debris. The supernatants were centrifuged at 16,000*g* for 20 min at 4 °C to pellet the mitochondria and to obtain a post-mitochondrial supernatant fraction.

Cytochrome c ELISA kit (Invitrogen, Mumbai) was used to estimate cytochrome c content in the post-mitochondrial supernatant fraction as per the manufacturer's instructions. Measurements were performed in duplicate, and cytochrome c content was analyzed at 450 nm (Bhoj et al. 2018).

Synergistic effect of piperidine compound and Michael chalcone compound

We attempted to test a possible synergism between piperidine compound and Michael adduct of chalcone for the inhibitory effect on folate metabolism and consequent DNA synthesis. Considering IC_{100} as a starting point, serial dilutions had been added along the ordinate and abscissa with an interval of 2 μM up to the lowest concentration (no drug) of both piperidine and chalcone compounds, respectively, in 96-well culture plates. One hundred Mf were added in each well, and volume was made up to 200 μL. Plates were incubated at 37 °C for 48 h under aerobic conditions. After incubation, the percentage loss of motility was recorded. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of individual drug that completely inhibited the motility. Synergism is more likely to be expressed when the ratio of the concentration of each drug to the MIC of that drug was the same for all components of the mixture. The ΣFICs (fractional inhibitory concentrations) were calculated as follows: ΣFIC = FIC A + FIC B, where FIC A is the MIC of drug A in the

combination/MIC of drug A alone and FIC B is the MIC of drug B in the combination/MIC of drug B alone. The combination was considered synergistic when the ΣFIC is ≤ 0.5, indifferent when the ΣFIC is > 0.5 to < 2, and antagonistic when the ΣFIC is ≥ 2 (Orhan et al. 2005).

Statistical analysis

The results were expressed as mean ± SD. After confirming the fulfillment of normality assumption of the data sets, for comparison of means of different parameters between the test compound and control, Student's *t* test was used. A p value less than 0.05 was considered significant.

Results

A total of 25 piperidine derivatives (synthesized and characterized previously; Agrawal et al. 2015) were screened in vitro with a dose up to 500 μM for each individual drug, out of which 4Q and 4H piperidine derivatives showed maximum antifilarial efficacy in terms of 100% loss of motility of Mf exposed to the drug (Table 1). All other drugs did not show considerable effect.

Next, we carried out molecular docking studies using AutoDock tool to predict how 4Q and 4H bind to the pre-validated structure of TS enzyme of *B. malayi* as well as its human counterpart (Fig. 1). As shown in Table 2, 4Q and 4H piperidine derivatives showed higher binding affinity towards *B. malayi* TS enzyme than its counterpart in the human host. Moreover, the observed inhibition constant showed significantly lower values. This inhibition constant represents the relative concentration required to achieve the inhibition of the proposed target.

Out of two compounds, 4H showed substantially significant thermodynamic parameters in favor of selective binding against the parasitic target. Consequently, the same result has been validated by in vitro experiments by assessing IC_{50}, IC_{100}, and LD_{50} of the 4H compound which were found to be 11.76 ± 0.24 μM, 16 ± 0.138 μM, and 150 ± 5.057 μM, respectively. The wider difference between IC_{50} and LD_{50} indicated the available therapeutic window.

As suggested by the bioinformatics analysis, effect of the proposed drug on parasitic target was validated by the antifilarial reversal study in terms of loss of motility. It showed significant reversal in the antifilarial activity as reflected by only a 12 ± 3.24% loss of motility of 4H treated parasites pre-treated with TS product i.e., thymidine, as compared with 93 ± 1.74% loss of motility in drug-treated parasite (Fig. 2).

In EB/AO fluorescent-staining experiment, untreated Mf remained green, indicating live parasites. Treatment with staurosporine (standard apoptosis inducer) and 4H compound at IC_{100} concentration resulted in orange-yellow stained Mf, reflecting a loss in cellular integrity due to apoptosis (Fig. 3a). The conversion of MTT to the colored formazan by active mitochondrial dehydrogenases is a known marker of functional mitochondria in a live cell. Exposure to 4H resulted in a significant reduction in purple color formation by more than 90% compared with untreated Mf, indicating severely compromised mitochondrial function (Fig. 3b). Significantly increased level of cytosolic cytochrome c in the 4H compound as well as staurosporine treated Mf but not in untreated Mf suggests impending apoptotic process due to mitochondrial damage (Fig. 3c). Further, 4H treated parasite showed comparable inhibition (38%) in PARP activity like that of positive control, staurosporine (40.86%) (Fig. 3d), considering untreated control as a reference of 100% uninhibited PARP activity.

Synergism between piperidine derivative, 4H, and Michael adduct of chalcone was further studied. The observed FIC was 0.47 ± 0.014, suggesting a significant synergistic effect.

Discussion

Initially, assessment of the antifilarial efficacy of synthetic library of piperidine compounds against *B. malayi* using Mf stage of the parasite was performed wherein 4Q and 4H showed a direct in vitro dose-dependent microfilaricidal activity at micro-molar concentration. In contrast to the conventional drug—DEC, these piperidine compounds showed direct microfilaricidal activity. As DEC fails to show antifilarial activity in vitro in the absence of host immune cells (Maizels and Denham 1992), it could not be used as a positive control. Presence of hydroxyl groups appeared as a notable common feature of both 4Q and 4H structures as displayed in structural detail of the molecules (Table 1). Other compounds in the series which lack the hydroxyl groups in analogous positions failed to record significant effect. Therefore, presence of hydroxyl groups might be contemplated as the pre-requisite for the antifilarial activity shown by these two compounds. Moreover, as anticipated, −OH groups of both 4Q and 4H were found to be involved in the formation of hydrogen bonds with parasitic TS protein as demonstrated in in silico results. As 4H showed thermodynamically favorable binding parameters to the target protein than 4Q, the former was selected for further study.

Although suitable for preliminary speculation, these virtual results must further be authenticated by apposite experimental results. Wider difference between IC_{50} and LD_{50} obtained by the in vitro experiments confirmed the safe therapeutic window for this agent as predicted by in silico study. Since thymidine synthesis is a vital metabolic reaction in DNA synthetic pathway, it might be expected to be conserved across species and evolutional hierarchy, raising an obvious concern about a nonspecific effect of its inhibitor on the host organism.

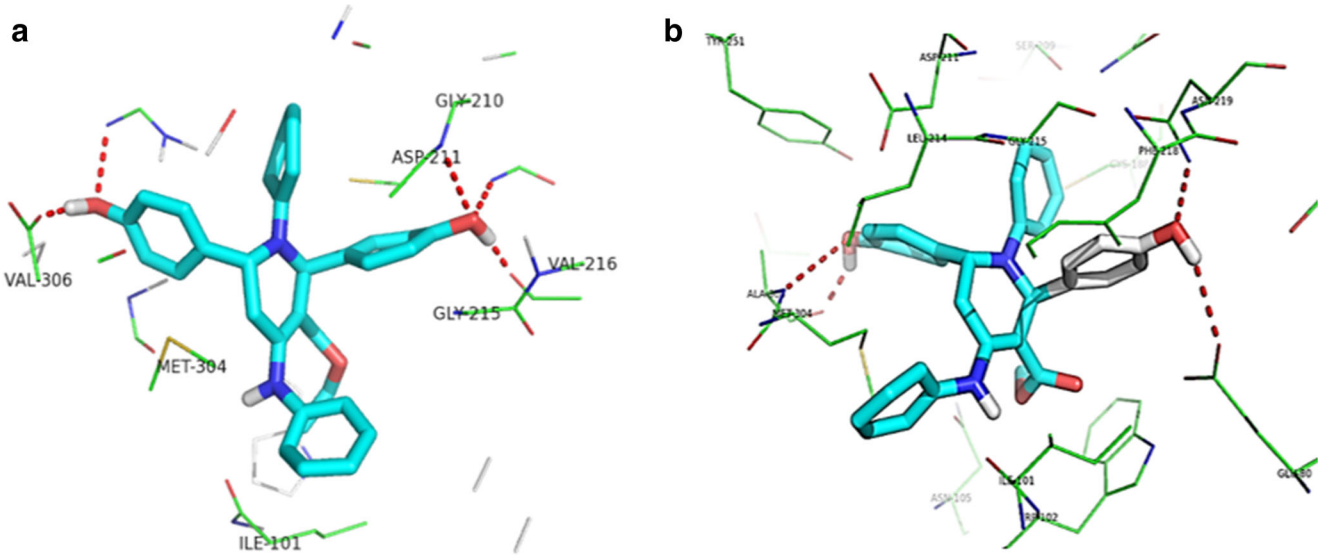


Fig. 1 Molecular docking of **a** 4Q and **b** 4H piperidine compounds against *Brugia malayi* thymidylate synthase (BmTS)

In this context, this large therapeutic index provides reasonable evidence in favor of its possible safe usage. Although a standard inhibitor of TS, 5-fluoro-2'-deoxyuridine acts effectively against certain human cancer (Longley et al. 2003), it failed to show any notable effect against the filarial Mf (data not shown). This probably indicates species-specific differential affinity of the binding domain of the target protein. Similar examples of species-specific antifolate agents are already well-known and are in respective clinical usage (Schweitzer et al. 1990).

In this study, selected piperidine was explored further for its therapeutic rationale against *B. malayi*. Possible inhibition of TS enzyme was contemplated to interfere with the synthesis of DNA by inhibiting the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) using methyl tetra-hydrofolate. Therefore, followed by in silico docking study, the in vitro reversal assay of piperidine-mediated effect, using thymidine, a product of this enzymatic reaction, was performed to authenticate such proposed inhibition. Interestingly, the antifilarial activity of piperidine compound was found to be reversed by approximately 80% in terms of observed loss of motility of Mf by pretreatment with thymidine. This result confirmed the possible implication of TS inhibition in the pharmacological rationale of piperidine derivatives used in this study.

The proposed inhibition of TS which plays a crucial step in the DNA synthesis can be envisaged to result into consequent cell cycle arrest at S phase followed by apoptosis. To test whether such apoptosis might occur due to proposed TS inhibition, EB/AO differential staining was performed. This staining reflects damage of membrane due to the cellular apoptotic process leading to a selective passage of dye to stain the cellular nuclei. While AO can stain nuclear DNA even by passing through an unbroken cell membrane, EB can only stain cells that have a damaged cell membrane. This leads to differential staining of viable cells to appear as green and apoptotic cells to fluoresce orange. Orange-red color fluorescence of both staurosporine- and piperidine-treated Mf was a characteristic for piperidine-induced apoptosis, which was not observed in untreated control Mf.

Mitochondria has a key role in the induction of apoptosis. Many researchers have demonstrated the involvement of mitochondria in the induction of apoptosis by TS inhibitors. TS inhibition specifically in L1210 leukemic cells has been shown to induce biphasic modification of mitochondrial membrane potential leading to caspase-dependent apoptosis (Sakoff and Ackland 2000). Release of cytochrome c in the cytosol is usually an upstream event before the activation of caspases, which is considered as a hallmark of apoptosis. Cytochrome c release from the mitochondria is an essential

Table 2 Free energy of binding (ΔGb) and estimated inhibition constant (Ki) calculated for piperidine compounds against thymidylate synthase target

Ligands	Free energy of binding		Inhibition constant (Ki)	
	B. malayi TS	*Homo sapiens* TS	*B. malayi* TS	*Homo sapiens* TS
4Q	− 9.86 kcal/Mol	− 8.4 kcal/Mol	59.37 nM	694.47 nM
4H	− 10.67 kcal/Mol	− 7.72 kcal/Mol	15.04 nM	2.19 μM

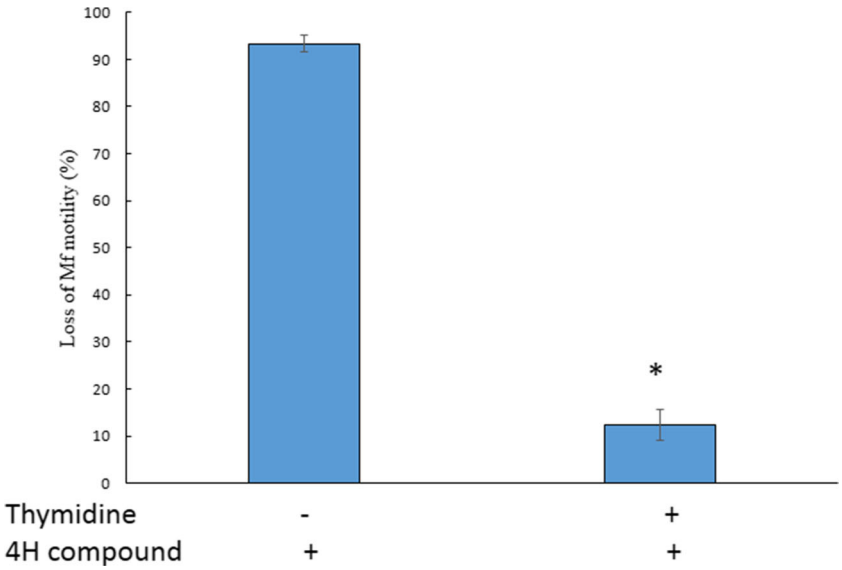


Fig. 2 Reversal of 4H compound–mediated loss of Mf motility by pre-incubation Mf with thymidine (100 μM). *p < 0.05 compared with 4H-only–treated Mf (positive control). Results are expressed as mean ± SD

feature of the induction of apoptosis by the standard TS inhibitor 5-fluoro-2'-deoxyuridine (Muñoz-Pinedo et al. 2004). In the present study, results showed significantly increased cytosolic cytochrome c level in both the 4H- and staurosporine-treated parasites as opposed to the untreated Mf. These results indicated initiation of the apoptotic process by the drug treatment. Concomitant decrease in formazan in MTT assay (which indicates loss of viability associated with dysfunction

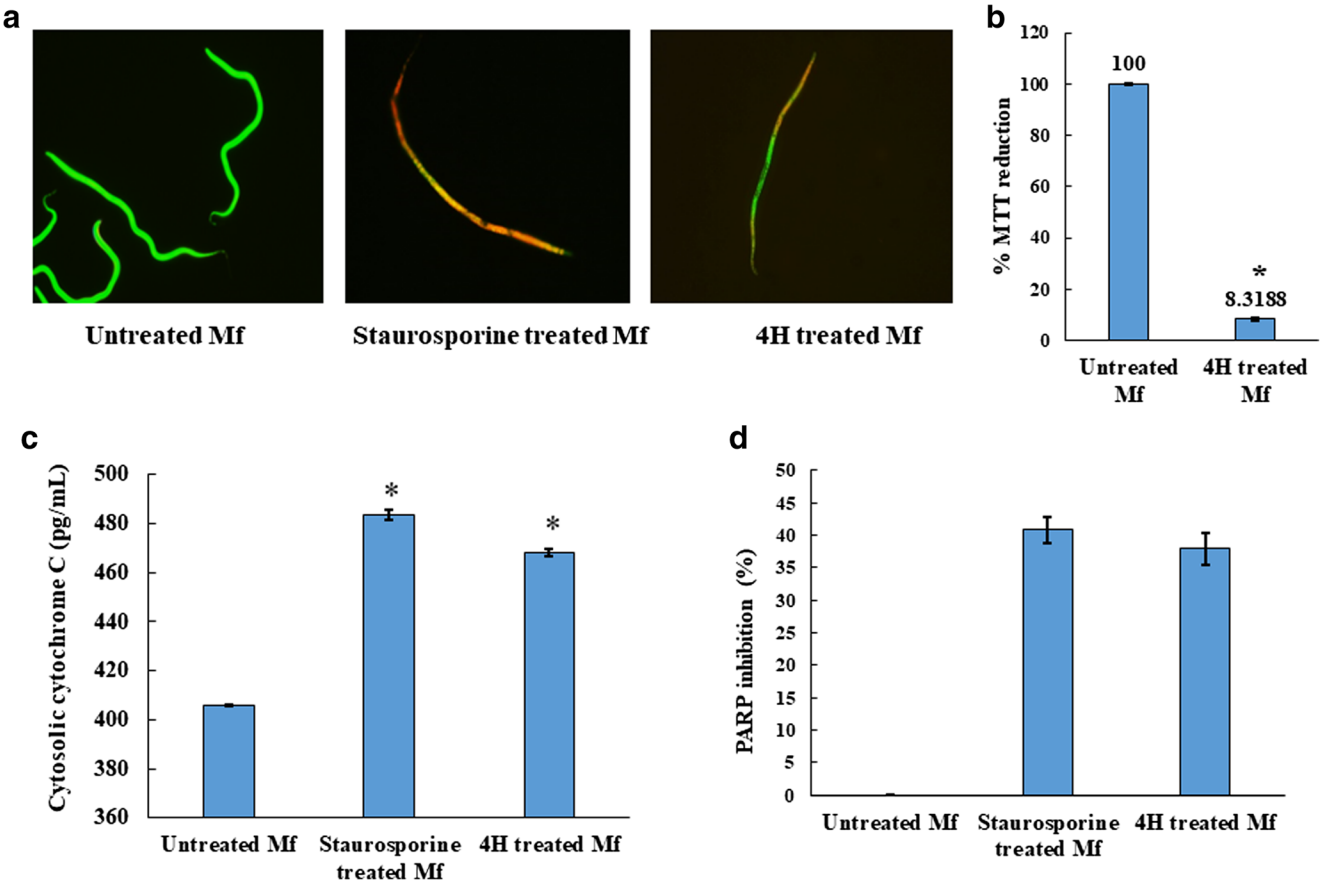


Fig 3 Assessment of involvement of induction of apoptosis by **a** Fluorescent EB/AO dual staining (× 40 magnified images), **b** MTT assay, **c** Cytochrome c ELISA assay, and **d** PARP colorimetric assay of untreated and 4H- or staurosporine (positive control)- treated Mf. Data is expressed as mean ± SD. *p < 0.05 compared with untreated Mf

of mitochondrial dehydrogenase) along with cytochrome c release provides corroborative evidence of mitochondrial damage in the drug treated Mf. Therefore, effective TS inhibition by the representative piperidine compound indicates possible induction of apoptosis by recruitment of caspases. The effect of the same was confirmed by the significant inhibition of PARP activity. PARP is a specific DNA repair enzyme, which acts as a substrate for activated caspase 3, that gets cleaved leading to a consequent PARP inhibition with ensuing apoptosis. Assuming PARP activity in untreated control parasite as a reference standard, demonstration of inhibition of the PARP enzyme by piperidine treatment comparable with staurosporine provided confirmatory proof of apoptosis. Therefore, with available evidence obtained in this study, piperidine can be contemplated to have significant antiparasitic potential by targeting TS.

Clinical experience shows that single-targeted therapy might develop drug resistance. For instance, targeting only a DHFR enzyme by standard antifolate, methotrexate is known to cause drug resistance in cancer chemotherapy (del-Campo et al. 2013). Thus, instead of inhibiting single target, if multiple enzymes of the same pathway like DHFR and TS enzyme of *B. malayi* are inhibited in tandem, the therapeutic effect might be expected to be more pronounced and also less amenable to the development of resistance. We have previously shown parasitic DHFR as a therapeutic target (Hande et al. 2015). Based on the structural similarity of chalcone derivatives with folate, N-(4-(4,4-dicyano-3-p-tolylbutanoyl)phenyl) benzene sulfonamide (a Michael chalcone adduct), had shown significant antifilarial potential in vitro at 38 μM, with significant inhibition of filarial DHFR protein (communicated elsewhere). With this perspective, we attempted to detect the evidence of synergism between Michael chalcone adduct and piperidine compound through possible multistep inhibition on DNA synthetic pathway. Our result confirming synergistic inhibition of the two pivotal enzymes in DNA synthesis, namely DHFR and TS working in tandem, by Michael adduct chalcone and piperidine compound, respectively, proved therapeutic implication of targeting parasitic DNA synthetic process. However, the effective combination for developing a suitable formulation with these inhibitors needs to be tested further for efficacy as well as safety.

Conclusion

It can be surmised that piperidine derivatives with hydroxyl substitution have great therapeutic potential for delivering antiparasitic effect through induction of apoptosis. This possibly involves the mitochondrial pathway due to the inhibition of thymidylate synthase. Therefore, with the available evidences obtained in this study, we conclude that parasitic DNA synthesis can be effectively targeted for therapeutic purpose. This might have obvious significance in developing a suitable antiproliferative strategy against a wide range of conditions with comparable pathogenesis.

Acknowledgments We would like to extend our tribute to the late Dr. MVR Reddy for his active contribution in this work.

Funding information This research work was supported by the funding received from Department of Biotechnology (DBT) (Project Id. BT/PR/4988/INF/22/155/2012), Government of India.

Compliance with ethical standards The animals used for the experimental purpose were approved by the Institutional Animal Ethics Committee (IAEC), MGIMS, Sevagram, Maharashtra, which follows the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) norms. Peripheral blood mononuclear cells (PBMCs; 1×10^6 cells/mL) of healthy human volunteers, of age 25 to 30 with informed consent, were collected and have been performed in accordance with the ethical recommendations of the Institutional Ethical Committee, MGIMS, Sevagram.

Conflict of interest The authors declare that they have no conflict of interest.

References

Agarwal A, Awasthi SK, Murthy PK (2011) *In vivo* antifilarial activity of some cyclic and acylic alcohols. Med Chem Res 20:430–434

Agrawal NR, Bahekar SP, Sarode PB, Zade SS, Chandak HS (2015) L-Proline nitrate: a recyclable and green catalyst for the synthesis of highly functionalized piperidines. RSC Adv 5:47053–47059

Ash LR, Riley JM (1970) Development of subperiodic *Brugia malayi* in the jird, *Meriones unguiculatus*; with notes on infections in other rodents. J Parasitol 9:123–128

Bahekar SP, Hande SV, Agrawal NR, Chandak HS, Bhoj PS, Goswami K, Reddy MV (2016) Sulfonamide chalcones: synthesis and *in vitro* exploration for therapeutic potential against *Brugia malayi*. Eur J Med Chem 124:262–269

Bhoj PS, Ingle RG, Goswami K, Jena L, Wadher S (2018) Apoptotic impact on *Brugia malayi* by sulphonamido-quinoxaline: search for a novel therapeutic rationale. Parasitol Res 1:1–4

del-Campo LS, Montenegro MF, Saez-Ayala M, Fernández-Pérez MP, Cabezas-Herrera J, Rodriguez-Lopez JN (2013) Cellular and molecular mechanisms of methotrexate resistance in melanoma. InMelanoma-From early detection to treatment 2013. InTechOpen. https://www.intechopen.com/books/melanoma-fromearly-detection-to-treatment/cellular-and-molecular-mechanisms-of-methotrexate-resistance-in-melanoma. Accessed 30 January 2013

Falco EA, Goodwin LG, Hitchings GH, Rollo IM, Russell PB (1951) 2: 4-diaminopyrimidines—A new series of antimalarials. Br J Pharmacol 6:185–200

Fries KM, Joswig C, Borch RF (1995) Synthesis and biological evaluation of 5-fluoro-2'-deoxyuridine phosphoramidate analogs. J Med Chem 38:2672–2680

Gupta S, Srivastava AK (2005) Biochemical targets in filarial worms for selective antifilarial drug design. Acta Parasitol 50:1

Hande S, Goswami K, Sharma R, Bhoj P, Jena L, Reddy MV (2015) Targeting folate metabolism for therapeutic option: a bioinformatics approach. Indian J Exp Biol 53:762–766

Houghton JA, Harwood FG, Tillman DM (1997) Thymineless death in colon carcinoma cells is mediated via fas signaling. Proc Natl Acad Sci U S A 94:8144–8149

Longley DB, Harkin DP, Johnston PG (2003) 5-fluorouracil: mechanisms of action and clinical strategies. Nat Rev Cancer 3:330

Mahalakshmi G, Balachandran V (2014) Molecular structure, vibrational spectra (FTIR and FT Raman) and natural bond orbital analysis of 4-Aminomethylpiperidine: DFT study. Spectrochim Acta A Mol Biomol Spectrosc 131:587–598

Maizels RM, Denham DA (1992) Diethylcarbamazine (DEC): immunopharmacological interactions of an anti-filarial drug. Parasitol 105:S49–S60

Mandvikar A, Hande S, Yeole P, Goswami K, Reddy MVR (2016) Therapeutic potential of novel heterocyclic thiazolidine compounds against human lymphatic filarial parasite: an *in vitro* study. Int J Pharm Sci Res 7:1480–1492

Muñoz-Pinedo C, Robledo G, López-Rivas A (2004) Thymidylate synthase inhibition triggers glucose-dependent apoptosis in p53-negative leukemic cells. FEBS Lett 570:205–210

Nelson RG, Rosowsky A (2001) Dicyclic and tricyclic diaminopyrimidine derivatives as potent inhibitors of *Cryptosporidium parvum* dihydrofolate reductase: structure-activity and structure-selectivity correlations. Antimicrob Agents Chemother 45:3293–3303

Orhan G, Bayram A, Zer Y, Balci I (2005) Synergy tests by E test and checkerboard methods of antimicrobial combinations against *Brucella melitensis*. J Clin Microbiol 43:140–143

Rao UR, Mehta K, Subrahmanyam D, Vickery AC (1991) *Brugia malayi* and *Acanthocheilonema viteae*: antifilarial activity of transglutaminase inhibitors in vitro. Antimicrob Agents Chemother 35:2219–2224

Rimington C (1934) *Psilocaulon absimile* NE Br. As a stock poison. S Afr J Sci 31:184–193

Sakoff JA, Ackland SP (2000) Thymidylate synthase inhibition induces S-phase arrest, biphasic mitochondrial alterations and caspase-dependent apoptosis in leukaemia cells. Cancer Chemother Pharmacol 46:477–487

Sanger I, Lammler G, Kimming P (1981) Filarial infection of *Mastomys natalensis* and their relevance for experimental chemotherapy. Acta Trop 38:277–288

Schweitzer BI, Dicker AP, Bertino JR (1990) Dihydrofolate reductase as a therapeutic target. FASEB J 4:2441–2452

Sharma RD, Bag S, Tawari NR, Degani MS, Goswami K, Reddy MVR (2013) Exploration of 2, 4-diaminopyrimidine and 2, 4-diamino-s-triazine derivatives as potential antifilarial agents. Parasitol 140: 959–965

Sharma V, Chitranshi N, Agarwal AK (2014) Significance and biological importance of pyrimidine in the microbial world. Int J Med Chem 2014:202784

Singh SK, Goswami K, Sharma RD, Reddy MV, Dash D (2012) Novel microfilaricidal activity of nanosilver. Int J Nanomedicine 7:1023–1030

World Health Organization. Lymphatic filariasis. Fact sheet updated on March 2017 http://www.who.int/mediacentre/factsheets/fs102/en/.