Homology Modeling of NAD⁺-Dependent DNA Ligase of the *Wolbachia* Endosymbiont of *Brugia malayi* and Its Drug Target Potential Using Dispiro-Cycloalkanones

Nidhi Shrivastava,^a Jeetendra K. Nag,^a Jyoti Pandey,^b Rama Pati Tripathi,^b Priyanka Shah,^c Mohammad Imran Siddiqi,^c Shailja Misra-Bhattacharya^a

Divisions of Parasitology,^a Medicinal and Process Chemistry,^b and Molecular and Structural Biology,^c CSIR-Central Drug Research Institute, Lucknow, Uttar Pradesh, India

Lymphatic filarial nematodes maintain a mutualistic relationship with the endosymbiont *Wolbachia*. Depletion of *Wolbachia* produces profound defects in nematode development, fertility, and viability and thus has great promise as a novel approach for treating filarial diseases. NAD⁺-dependent DNA ligase is an essential enzyme of DNA replication, repair, and recombination. Therefore, in the present study, the antifilarial drug target potential of the NAD⁺-dependent DNA ligase of the *Wolbachia* symbiont of *Brugia malayi* (wBm-LigA) was investigated using dispiro-cycloalkanone compounds. Dispiro-cycloalkanone specifically inhibited the nick-closing and cohesive-end ligation activities of the enzyme without inhibiting human or T4 DNA ligase. The mode of inhibition was competitive with the NAD⁺ cofactor. Docking studies also revealed the interaction of these compounds with the active site of the target enzyme. The adverse effects of these inhibitors were observed on adult and microfilarial stages of *B. malayi* *in vitro*, and the most active compounds were further monitored *in vivo* in jirds and mastomys rodent models. Compounds 1, 2, and 5 had severe adverse effects *in vitro* on the motility of both adult worms and microfilariae at low concentrations. Compound 2 was the best inhibitor, with the lowest 50% inhibitory concentration (IC₅₀) (1.02 μM), followed by compound 5 (IC₅₀, 2.3 μM) and compound 1 (IC₅₀, 2.9 μM). These compounds also exhibited the same adverse effect on adult worms and microfilariae *in vivo* (*P* < 0.05). These compounds also tremendously reduced the wolbachial load, as evident by quantitative real-time PCR (*P* < 0.05). wBm-LigA thus shows great promise as an antifilarial drug target, and dispiro-cycloalkanone compounds show great promise as antifilarial lead candidates.

Human lymphatic filariasis (LF) continues to be a major public health problem as a vector-borne communicable disease in many tropical and subtropical areas of the world. It is caused by the nematode parasites *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori*, affecting >1.3 billion people in 81 countries worldwide, and several millions more are at risk of infection. The mainstay of filarial disease control has been a limited number of drugs, such as diethylcarbamazine, albendazole, and ivermectin, which are principally microfilaricidal, thus necessitating the need for repeated administrations (1, 2). Furthermore, signs of emerging drug resistance are becoming increasingly apparent, especially against albendazole and ivermectin (3–5). There is an urgent need to develop novel drugs (particularly macrofilaricidal drugs) against LF, and also, renewed efforts are required to identify suitable antifilarial drug targets. Numerous lines of evidence, from both laboratory experiments and human trials, show that depletion of *Wolbachia* in filarial parasites by the use of antibiotics (e.g., doxycycline and tetracycline) can kill adult worms in addition to impairing embryogenesis, the output of microfilariae (MF), and worm development (6, 7). Furthermore, *Wolbachia* has been identified as a major contributor to inflammatory host pathology and severe adverse reactions to antifilarial chemotherapy (8, 9). Therefore, *Wolbachia* can be used as a drug target and holds great promise for therapeutic options. More research is therefore desired to explore new biochemical pathways and new enzymes in the *Wolbachia* life cycle that are involved in *Wolbachia* growth and development. Once identified, the specific enzyme inhibitors may be used in antifilarial therapy. The completed genome sequence of the *Wolbachia* endosymbiont of *B. malayi* (wBm) enabled genome-wide mining for new drug targets and provided a founda-

tion for rational drug design (10). A few wolbachial proteins are already being explored as antifilarial drug targets, such as the enzymes involved in heme biosynthesis and lipoprotein biosynthesis and the cell division protein FtsZ (11–13).

An NAD⁺-dependent DNA ligase is an indispensable enzyme in prokaryotes for many fundamental processes in DNA metabolism, including the linkage of Okazaki fragments during replication, recombination processes, and repair pathways requiring re-synthesis of DNA (14, 15). Gene knockout and other studies have shown that NAD⁺-dependent DNA ligases are essential enzyme in several prokaryotes (16–18). Thus, all prokaryotes use an NAD⁺-dependent DNA ligase, whereas all human DNA ligase activities are ATP dependent, providing a selective drug target for *Wolbachia* endosymbionts. The dispiro-cycloalkanones are a new class of compounds that were recently discovered to be potent inhibitors of the NAD⁺-dependent DNA ligase of *Mycobacterium tuber-*

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Address correspondence to Shailja Misra-Bhattacharya, shailja_cdri@rediffmail.com.

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TABLE 1 Influence of dispiro-cycloalkanones on the nick-closing activity and cohesive-end ligation activity of wBm-LigA^a

Compound	Chemical structure of dispiro-cycloalkanone	MIC (μM)			
		wBm-LigA		Nick-closing activity of T4 Lig	Nick-closing activity of Hlig I
		Nick-closing activity	Cohesive-end ligation activity		
1		0.5	0.25	>100	>100
2		0.05	0.05	>100	>100
3		2.5	0.5	>100	>100
4		1.0	0.25	>100	>100
5		0.1	0.1	>100	>100
6		2.0	0.5	>100	>100
7		5.0	1.0	>100	>100

^a The influence of dispiro-cycloalkanones on the nick-closing activity and cohesive-end ligation activity of wBm-LigA was analyzed *in vitro* and contrasted with the effect on the nick-closing activity of purified ATP-dependent human DNA ligase (Hlig I) and commercially prepared T4 DNA ligase (T4 Lig).

culosis and demonstrated very promising antitubercular activities (19). In the present investigation, we tested the inhibitory activity of dispiro-cycloalkanones on a recombinant NAD⁺-dependent DNA ligase of wBm (wBm-LigA), followed by validation of wBm-LigA as an antifilarial drug target by using these enzyme inhibitors.

MATERIALS AND METHODS

Synthesis of dispiro-cycloalkanones. The dispiro-cycloalkanones (compounds 1 to 7) were prepared by employing our previously reported protocol involving the Corey-Chaykovsky reaction of bis-benzylidene cycloalkanones with trimethylsulfoxonium iodide (TMSOI) in the presence of NaOH as the base and tetrabutylammonium bromide (TBAB) as the phase transfer catalyst (20). For *in vitro* antifilarial studies, the compounds were prepared in dimethyl sulfoxide (DMSO), and for *in vivo* studies, the compounds were prepared in phosphate-buffered saline (PBS) with 0.1% (vol/vol) Tween 20.

Expression and purification of recombinant wBm-LigA. Expression and purification of wBm-LigA (GenBank accession number

AAW71136.1) were done as described previously (21). Briefly, a recombinant construct of wBm-LigA in the pTZ57R/T vector was subcloned into the pET28a(+) expression vector (Novagen) and maintained in *Escherichia coli* DH5 α cells. For overexpression, the recombinant protein wBm-LigA-pET28a construct was transformed into *E. coli* Rosetta cells (Novagen, USA). Bacterial cells carrying the wBm-LigA-pET28a construct were grown at 37°C until the optical density at 600 nm (OD₆₀₀) reached 0.6, and the culture was then induced with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG; Sigma) for 5 h at 25°C. The bacterial cells were pelleted at 3,000 $\times$ g for 10 min at 4°C and resuspended in buffer A (20 mM Tris-HCl, 250 mM NaCl, and 10 mM imidazole [pH 8.0]). The bacterial cells were lysed in the presence of lysozyme (1 mg/ml) and Triton X-100 (0.1%, vol/vol), and the lysate was sonicated to reduce viscosity and then centrifuged at 12,000 $\times$ g for 30 min at 4°C. The soluble fraction obtained after centrifugation was applied onto a Ni-nitrilotriacetic (Ni-NTA) agarose column (Qiagen, USA) that was preequilibrated with buffer A. The column was washed with wash buffer B (20 mM Tris-HCl, 250 mM NaCl, 50 mM imidazole [pH 8.0]), followed by elution of the recombinant

FIG 1 (A) Purification of wBm-LigA by affinity chromatography using a nickel column. Lane M, standard protein molecular mass marker; lane 1, soluble *E. coli* proteins following induction with 1.0 mM IPTG at 25°C for 5 h; lane 2, flowthrough from the nickel column; lane 3, washthrough from the column prior to elution; lane 4, elution of His-tagged purified recombinant wBm-LigA at an imidazole concentration of 250 mM. (B) Western blot analysis. Lane M, standard protein molecular mass marker; lane 1, His-tagged wBm-LigA. (C) Purification of human DNA ligase I (HliG I) by affinity chromatography using a nickel column. Lane M, standard protein molecular mass marker; lane 1, soluble *E. coli* proteins; lane 2, flowthrough from the nickel column; lanes 3 to 5, washthrough; lane 4, eluted HliG I. (D) Western blot analysis. Lane M, standard protein molecular mass marker; lanes 1 and 2, human ligase A.

protein with buffer C (20 mM Tris-HCl, 250 mM NaCl, 250 mM imidazole [pH 8.0]). The protein was dialyzed against buffer B followed by buffer D (20 mM Tris-HCl, 250 mM NaCl). The protein content was estimated with the Bradford reagent (22), using bovine serum albumin (BSA) as the standard.

Expression and purification of human DNA ligase I. Plasmid constructs of human DNA ligase I (HliG I) (GenBank accession number M36067) were transformed into *E. coli* strain BL21. Individual colonies were grown to mid-log phase in L broth supplemented with 50 µg/ml carbenicillin and then induced with 100 µM isopropyl-β-D-thiogalactopyranoside for 4 h at 20°C. Cells were harvested by centrifugation at 1,000 × g for 5 min, washed once in phosphate-buffered saline, and resuspended in buffer A (50 mM HEPES-NaOH [pH 8.0], 500 mM NaCl, 0.1 mM EDTA, and 10% glycerol) supplemented with 1 mM dithiothreitol (DTT) and protease inhibitors. Cells were lysed by sonication, and debris was removed by centrifugation at 14,000 × g for 20 min. The extracts were loaded onto a column of Ni²⁺-nitrilotriacetic acid resin (Qiagen) equilibrated with buffer A. The column was washed with buffer A containing 1 mM imidazole and 1 mM DTT and then step-eluted with increasing concentrations of imidazole in buffer B (50 mM HEPES-NaOH [pH 7.0], 100 mM NaCl, 0.1 mM EDTA, 10% glycerol, and 1 mM DTT). HliG I was eluted with 80 mM imidazole (23). The protein content was estimated by using the Bradford reagent, and enzymatic nick activity assays were later performed with this recombinant protein.

Enzyme assays to determine the *in vitro* inhibition of the nick-closing activity of wBm-LigA by dispiro-cycloalkanones. To determine that dispiro-cycloalkanones are specific inhibitors of wBm-LigA, the effect of dispiro-cycloalkanones (compounds 1 to 7) on the nick-closing activity of the recombinant wBm-LigA enzyme was determined by a sealed duplex assay as described previously (24), in the presence of various concentrations of compounds. This ligase assay format was based on the ligation of a fluorescein-labeled oligonucleotide to a biotinylated oligonucleotide

within a double-strand duplex hybridized on a streptavidin-coated microtiter plate. The amount of fluorescence captured following denaturation of DNA duplex quantifies successful ligation. The three oligonucleotides used, *viz.*, 5'-fluorescein-AAAATGACCCCC-3' (20 pmol), 5'-CC AGACAACGTCG-biotin-3' (20 pmol), and 5'-GACGTTGTCTGGGGG GGGTCATTTT-3' (20 pmol) (Eurogentec, Germany), were suspended in buffer H (5× SSC [0.75 M NaCl, 0.075 M sodium citrate], 0.1% [wt/vol] BSA, 0.1% [vol/vol] Triton X-100) and hybridized on Strepta Well High-Bind plates (Thermo Scientific, USA) for 90 min at 20°C to produce the nicked substrate. Wells were washed with 400 µl of wash buffer [20 mM Tris-HCl (pH 8.0), 10 mM (NH₄)₂SO₄, 10 mM MgCl₂, 1.2 mM EDTA, and 1 mM DTT]. Ligation was performed for 90 min at 25°C in 150 µl of wash buffer, to which wBm-LigA and 26 µM NAD⁺ were added. This was followed by denaturation in 0.5% (wt/vol) SDS–0.5 M NaOH for 1 min. Fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 525 nm after washing the wells twice with 300 µl of TBS-T (Tris-buffered saline with 0.05% [vol/vol] Tween 20) and 0.1% (wt/vol) BSA. For inhibition assays, the compounds listed in Table 1 were added at an initial concentration of 100 µM, and the concentrations were lowered subsequently to determine the MICs of compounds. To determine the specificity of these compounds, inhibition assays were also performed by using human DNA ligase I and bacteriophage T4 DNA ligase (Invitrogen, USA) with the aid of a sealed duplex assay format as described above for wBm-LigA, with the only difference being that ATP was used as a cofactor in place of NAD⁺.

To elucidate the molecular mechanism of inhibition of wBm-LigA by dispiro-cycloalkanone, the nick-sealing reaction was performed with various concentrations of NAD⁺ and dispiro-cycloalkanone.

Inhibition of the cohesive-end ligation activity of wBm-LigA by dispiro-cycloalkanones. Inhibition of the cohesive-end ligation activity of wBm-LigA by dispiro-cycloalkanones was determined according to methods described previously by Muerhoff et al. (25). The standard reac-

FIG 2 Effects of different concentrations of dispiro-cycloalkanone compounds on the cohesive-end ligation activity of wBm-LigA. Cohesive ends of fragment 1 and fragment 4 of lambda DNA digested with BstEII were used as the substrate, and their ligation into the 14-kb ligated product was analyzed on a 1.0% agarose gel. Compound 1 completely inhibited the cohesive-end ligation activity of wBm-LigA at a 0.25 μ M concentration, compound 2 inhibited activity at 0.05 μ M, compound 4 inhibited activity at 0.25 μ M, and compound 5 inhibited activity at 0.1 μ M. The control lane denotes the control reaction, in which no enzyme was added.

tion mixture (20 μ l) contained the wBm-LigA enzyme in a solution containing 20 mM Tris-Cl (pH 8.0), 100 mM KCl, 10 mM MgCl₂, 26 μ M NAD⁺, 10 mM DTT, and 500 ng of BstEII-predigested lambda DNA (New England BioLabs, United Kingdom). For inhibition assays, various concentrations of compounds (0.5 to 0.0125 μ M) were added to the reaction mixture.

The reaction mixture was incubated at 25°C for 60 min, and the reaction was stopped by the addition of 4 μ l of 50 mM EDTA, followed by heating at 70°C for 5 min and subsequent chilling on ice for 5 min. The mixture was run on a 1.0% (wt/vol) agarose gel, and band intensities were determined by measuring UV fluorescence using digital images captured by the ChemiDoc system and analyzed by using the QuantityOne version 4 software package (Bio-Rad). Activity values were calculated by measuring the net intensity of the product band relative to the intensity of a nonsubstrate fragment (fragment 7).

Molecular modeling. The server for homology detection and structure prediction by hidden Markov model (HMM) comparisons, HHpred (<http://toolkit.tuebingen.mpg.de/hhpred>), was used for protein homology analysis and structure prediction. HHpred constructs a hidden Markov model based on a profile using query sequences against known databases to search for the closest possible homolog with an HMM profile-profile comparison. The Protein Data Bank (PDB) was chosen for profile analysis and homolog searching. The top-scoring PDB entry was selected as a template for further model building. The structure of the query sequence was constructed by using Modeler 9.11 interfaced with the HHpred server based on the template DNA ligase from *E. coli* (PDB accession number 4GLX). The model was further uploaded to the SAVS server for validation purposes. The model constructed by the HHpred server was further subjected to energy minimization using GROMACS 4.5 by the steepest-descent method, with an initial step size of 0.01 nm and a maximum of 5,000 steps. The minimization procedure was performed in explicit water by using the amber03 force field in the tip4p water model with periodic boundary conditions surrounded by an octahedron extended 10 Å from the protein surface. Electrostatics were treated by using the particle mesh Ewald (PME) method, using a cutoff of 1.0 for long-

range van der Waals and columbic forces. Minimization was converged within the allowed number of steps. The stereochemical qualities were checked by Verify-3D and Errat. For docking studies, coordinates of the DNA molecule co-complexed with the template protein were included in the model after superimposition of the template and model proteins.

Docking studies. Structures of inhibitors were built and minimized in Sybyl, according to the simulated annealing protocol for optimization of the three-dimensional (3D) structures of inhibitors, using 500 cycles of heating to 700 K for 1,000 fs and annealing to 200 K in 1,000 fs. Autodock4 was used for docking of the inhibitor with the target enzyme. Kollman charges were assigned with 40-by-40-by-40 grid points with a 0.375-Å spacing. Fifty autodock runs were performed for each inhibitor.

Animals and parasite isolation. Adult worms and MF of *B. malayi* were recovered aseptically from the peritoneal cavity of jirds within 120 to 150 days of intraperitoneal (i.p.) inoculation of *B. malayi* infective larvae (L3) (26). Six-week-old male jirds and *Mastomys coucha* (mastomys) rodents, which were maintained under proper housing conditions at the Laboratory Animal Division of the CSIR-Central Drug Research Institute (CDRI), Lucknow, India, and fed on a standard pellet diet and water *ad libitum*, were used in this study. Jirds were transplanted with adult *B. malayi* parasites and mastomys rodents were inoculated subcutaneously with 100 *B. malayi* L3 for *in vivo* studies. The Animal Ethics Committee of the CDRI duly constituted under the provisions of the CPCSEA (Committee for the Purpose of Control and Supervision on Experiments on Animals), Government of India, approved the animals and the animal experimental procedures. This study bears Institutional Animal Ethics Committee (IAEC) approval no. IAEC/2011/145, dated 7 March 2012.

Effect of dispiro-cycloalkanones on *B. malayi* adult worms and MF *in vitro*. Since filarial *Wolbachia* cannot be cultured *in vitro*, the effects of DNA ligase inhibitors belonging to the dispiro-cycloalkanone class were investigated by using *B. malayi* adult worms *in vitro*, where the process of compound exposure can be more carefully controlled. Two living *B. malayi* adult female worms were washed extensively with RPMI 1640 medium

FIG 3 Mode of inhibition of wBm-LigA with respect to NAD^+ by compound 2. (A) Lineweaver-Burk plot of the activity of wBm-LigA measured in the presence of different concentrations of NAD^+ (1 to 30 μM) and compound 2 at concentrations of 40 nM, 20 nM, 10 nM, 5 nM, and 0 nM. The graph is plotted between $1/V$ and $1/\text{NAD}^+$, where V is the velocity of the reaction expressed as micromolar per minute. The data clearly indicated competitive binding between NAD^+ and compound 2. (B) Determination of the inhibitory constant (K_i) of the inhibitor by plotting the different concentrations of compound 2 (0 to 40 nM) and their respective apparent K_m values ($K_m \text{ app}$) on a graph and then extrapolating the linear line to the x axis. The $-K_i$ value is marked with an arrow.

supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin sulfate, and a neomycin mixture; Sigma, USA). Worms were placed individually into a 48-well plate containing 1.0 ml medium. The plate was incubated at 37°C in a CO_2 incubator. The medium was replaced with fresh medium containing the same concentration of each compound on every alternate day up to day 9. Worms were exposed to different dispiro-cycloalkanone compounds, starting with a 100 μM concentration and subsequently lowering the concentrations to determine the 100% lethal concentration (LC_{100}) and the 50% inhibitory concentration (IC_{50}) (27). At the end of the test period, the worms were processed for a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay as described previously (28). In the case of MF, 20 live MF/well were placed into a 96-well plate containing 200 μl medium in duplicate. MF were also exposed to dispiro-cycloalkanones at various concentrations in the same way as that described above for adults, and the effect on motility was microscopically assessed.

An *in vitro* cytotoxicity assay with all the compounds was carried out for the assessment of CC_{50} (50% cytotoxicity concentration) values according to methods described previously by Misra et al. (27). The selectivity index (SI) for the use of compounds safely in animals was then calculated as $\text{CC}_{50}/\text{IC}_{50}$. Compounds with an SI of ≥ 10 are considered to be safe.

In vivo antifilarial activity of dispiro-cycloalkanones in *Meriones unguiculatus*. Six-week-old male jirds were used for *in vivo* studies. Fourteen female and seven male adult worms of *B. malayi* were transplanted intraperitoneally into jirds, and the compounds (compounds 1, 2, and 5) that were determined to be most effective *in vitro* were monitored *in vivo* in jirds. These compounds were administered at 10 mg/kg of body weight for seven consecutive days by the i.p. route to observe the direct effect of the inhibitors on intraperitoneally transplanted worms to obtain an accurate assessment of the adulticidal effect. At the end of the observation period (on day 52), the treated and control jirds were euthanized, and the worms were recovered from the peritoneal cavity. These worms were examined for their motility and viability. All surviving females were teased individually in a drop of saline, and the condition of the embryonic stages in the uteri was examined microscopically. Any abnormality or death/distortion detected in the uterine contents, including oocytes, eggs, and MF, was considered a sterilization effect, and the percent sterilization was assessed (27). The effect of inhibitors on MF load and motility was also determined by observing 10 μl of the peritoneal fluid collected from each jird under a microscope. The experiment was repeated.

Real-time PCR for determining the *Wolbachia* load. For quantification of the *Wolbachia* load in adult worms recovered from the control and treated jirds, genomic DNA was extracted from the whole parasite by

FIG 4 Three-dimensional homology model of wBm-LigA constructed by using Modeler 9.11. (A) Superimposition of wBm-LigA (model) (black) with the DNA ligase from *E. coli* (PDB accession number 4GLX) (template) (gray). (B) Homology model structure of wBm-LigA. The root mean square deviation between the C-alpha values of the two structures is 0.795 Å.

FIG 5 *In silico* molecular docking of compound 1 (A), compound 2 (B), compound 3 (C), and compound 4 (D) (in black) with the homology model of the target enzyme (in gray).

using a genomic DNA isolation kit (Invitrogen), and real-time PCR targeting the *ftsZ* gene (NCBI gene identification number 3266907) of *Wolbachia* was performed according to methods described previously (29). Briefly, the 20- μ l reaction mixture consisted of 1 μ g of genomic DNA from each group, 1 μ M concentrations of a forward primer (5'-AAA GCA ACA AGA GAG GCA AGA G-3') and a reverse primer (5'-AAG TTC CGC AAT ACG CAT ACG-3'), and 10 μ l (2 $\times$) of LightCycler-FastStart DNA Master SYBR green I (TaKaRa, Japan). Amplification involved incubation at 95°C for 10 min for initial denaturation, followed by 45 cycles consisting of (i) denaturation at 95°C for 30 s, (ii) annealing at 60°C for 40 s, (iii) extension at 72°C for 40 s, and (iv) fluorescence acquisition (530 nm) at the end of the extension step. The reactions were performed in triplicate, and threshold cycle (C_T) values were determined with LightCycler software (version 1.5), using the second-derivative method. The difference between the average C_T value for treated worms

and that for control adult worms is indicated as a ΔC_T value (ΔC_T value = C_T of treated worms - C_T of control worms). The ΔC_T value is proportional, with an exponential relationship, to the difference in *Wolbachia* contents (30). The *Wolbachia* load can also be denoted alternatively in terms of *ftsZ* copy numbers in treated and control adult worms. The copy number was calculated from a standard curve. The standard curve includes a plot of the C_T values of the known plasmid copy number (PCN) of a 10-fold serially diluted plasmid containing the *ftsZ* gene and was generated by linear regression of the plotted points. The *ftsZ*-containing plasmid was constructed by amplifying the *ftsZ* gene from genomic DNA of adult worms and subsequently cloning it into the pTZ57R/T vector according to the manufacturer's instructions (Invitrogen, USA). The concentration of the plasmid was determined by NanoDrop analysis, and the corresponding copy number was calculated according to the equation described previously by Whelan et al. (31).

FIG 6 *In silico* molecular docking of compound 5 (A), compound 6 (B), and compound 7 (C) (in black) with the homology model of the target enzyme (in gray).

***In vivo* antifilarial activity of dispiro-cycloalkanones in *Mastomys coucha*.** *Mastomys coucha* infected subcutaneously with *B. malayi* L3 acted as a secondary screening model. In *M. coucha*, adult worms are established in various tissues, such as heart, lungs, and testes, after subcutaneous

inoculation of L3, thus simulating a natural human LF infection. L3 were recovered from *Aedes aegypti* mosquitoes fed on donor mastomys 9 days earlier (32). Animals were monitored for microfilaremia by using 10 μ l of tail blood between noon and 1 p.m. from day 120 onwards, and those infected 5 to 8 months earlier that showed a progressive rise in microfilaremia were selected for treatment. Five mastomys were administered compounds 1, 2, and 5 (i.p. route at 10 mg/kg for 7 days), which possessed a maximum inhibitory effect on wBm-LigA in *in vitro* enzymatic assays and also revealed significant mortality of intraperitoneally transplanted worms in jirds. The inhibitors were administered intraperitoneally so that the activity was not missed in case the compound was poorly absorbed through the intestinal tract. To observe microfilaricidal activity, thick blood smears of 10 μ l tail blood from treated and untreated animals were made just before the start of treatment, i.e., on day 0, and on days 8, 15, 30, 60, and 90 after the initiation of treatment. The percent change in microfilaremia at each time point over the pretreatment level was evaluated. Animals infected under identical conditions that received only PBS served

TABLE 2 Docking energies of various dispiro-cycloalkanone compounds

Compound	Docking energy (kcal/mol)
1	-6.64
2	-9.19
3	-6.98
4	-7.37
5	-5.82
6	-6.84
7	-7.36

TABLE 3 *In vitro* antifilarial assay of dispiro-cycloalkanones against *Brugia malayi* adult worms and MF

Compound	Concn (μ M)	Adult motility score ^a	% inhibition in MTT reduction assay over control	MF motility score ^a
1	10	D	77.6	D
	5	1+	38.6	2+
	2.5	2+	30.8	2+
	1.25	3+	13.1	3+
2	5	D	83.6	D
	2.5	1+	75.2	D
	1.25	1+	37.1	D
	0.625	2+	28.3	1+
3	40	D	55.8	D
	20	2+	33.2	1+
	10	3+	28.9	2+
	5	3+	21.4	3+
4	20	D	57.8	D
	10	1+	35.6	D
	5	2+	23.1	D
	2.5	2+	17.9	2+
5	10	D	75.1	D
	5	1+	44.5	D
	2.5	2+	31.0	2+
	1.25	2+	25.1	3+
6	40	D	68.6	D
	20	2+	41.7	1+
	10	2+	36.9	2+
	5	3+	25.2	3+
7	75	D	58.7	D
	40	2+	36.3	1+
	20	3+	23.2	2+
	10	3+	15.0	2+

^a The reduction in the motility of the parasites was scored as follows: 4+ for 0% reduction, 3+ for 1 to 49% reduction, 2+ for 50 to 74% reduction, 1+ for 75 to 99% reduction, and D for 100% reduction.

as controls. At the end of the observation period (on day 91), the treated and control mastomys were euthanized, and various tissues (lungs, heart, testes, and lymph nodes) were teased gently in PBS (pH 7.2) to recover adult parasites. The experiment was repeated.

Statistical analysis. Data were analyzed by Dunnett's multiple-comparison test using one-way analysis of variance (ANOVA) with the help of PRISM 5.0 statistical software. A *P* value of <0.05 was considered significant.

RESULTS

Dispiro-cycloalkanones specifically inhibit the nick-closing activity of the recombinant enzyme wBm-LigA. The inhibition of the nick-closing activity of purified wBm-LigA by dispiro-cycloalkanones is described in Table 1. The results demonstrated that these compounds inhibited the nick-closing activity of wBm-LigA at low concentrations. Compounds 2 and 5 appeared to be the most effective inhibitors of wBm-LigA (MICs of 0.05 μ M and 0.1 μ M, respectively), followed by compound 1 (MIC of 0.5 μ M). Compounds 3, 4, 6, and 7 had MIC values of 2.5 μ M, 1.0 μ M, 2.0 μ M, and 5 μ M, respectively. On the other hand, all these com-

TABLE 4 *In vitro* evaluation of LC₁₀₀ and IC₅₀ values for adult *Brugia malayi* worms and MF

Compound	Adult		MF	
	LC ₁₀₀ (μ M)	IC ₅₀ (μ M)	LC ₁₀₀ (μ M)	IC ₅₀ (μ M)
1	10	2.9	10	4.1
2	5	1.02	1.25	0.35
3	40	17.7	40	6.1
4	20	5.8	5.0	1.8
5	10	2.3	5.0	2.2
6	40	15.0	40	12.4
7	75	35.4	75	23.5

pounds did not possess any inhibitory effect on the nick-closing activity of ATP-dependent human or bacteriophage T4 DNA ligase, even at concentrations of up to 100 μ M (Table 1). Thus, these findings clearly demonstrated that dispiro-cycloalkanones are specific inhibitors of wBm-LigA.

Dispiro-cycloalkanones inhibit the cohesive-end ligation activity of wBm-LigA. All the dispiro-cycloalkanones tested inhibited the ability of recombinant wBm-LigA to ligate the cohesive ends of BstEII-digested lambda DNA. Compounds 1, 2, 4, and 5 were most effective in inhibiting the cohesive-end ligation activity of wBm-LigA (Fig. 1). Compound 2, which was most effective in inhibiting the nick-closing activity of wBm-LigA, demonstrated complete inhibition of the cohesive-end ligation activity of wBm-LigA at 0.05 μ M, followed by compound 5, which completely inhibited this reaction at 0.1 μ M. Compounds 1 and 4 exhibited complete inhibition at a 0.25 μ M concentration (Fig. 1). Compounds 3 and 6 showed complete inhibition at 0.5 μ M, and compound 7 showed complete inhibition at 1.0 μ M (Table 1).

Dispiro-cycloalkanones inhibit nick-closing activity by competitive inhibition with NAD⁺. For determining the mechanism of inhibition of dispiro-cycloalkanones, a nick-closing activity assay was performed with various concentrations of inhibitors and the NAD⁺ cofactor. The dispiro-cycloalkanones inhibited the nick-closing activity of wBm-LigA by competitive inhibition with NAD⁺. This is illustrated in Fig. 2A by taking the example of compound 2, which was the most effective inhibitor with the lowest MIC. In the absence of compound 2, a *K_m* of 5.21 μ M for NAD⁺ was determined, which is in good agreement with our previously reported data (14). However, in inhibition studies, with an increase in the amount of NAD⁺ from 1 μ M to 30 μ M in the presence of various concentrations of compound 2 (0 to 40 nM), the kinetics indicated competitive inhibition, as visualized in a double-reciprocal plot, as the *V_{max}* remained the same before and after the addition of compound 2. Linear regression using the apparent *K_m* value (*K_{m app}*) leads to an inhibitory constant (*K_i*) of ~0.035 μ M (Fig. 1B). These findings strongly suggest that the binding site of the dispiro-cycloalkanones overlaps that of the cofactor NAD⁺ at least partially.

Structure prediction. The HHpred-derived score suggests that the DNA ligase from *E. coli* (PDB accession number 4GLX) is the closest homolog of wBm-LigA. Subsequent alignment of the wolbachial enzyme with the sequence of the *E. coli* protein from which the structure reported under PDB accession number 4GLX is derived showed 40.78% identity. The 3D homology model of wBm-LigA is presented in Fig. 3. The modeled structure shows 93.1% of residues in the core region, 4.9% in the allowed region,

TABLE 5 *In vivo* activities of specific inhibitors in transplanted adult *Brugia malayi* worms

Compound	Dose (mg/kg) × days	No. of animals × no. of experiments	Avg no. of worms recovered ± SD			% reduction in adult worm recovery over control	Mean % sterilization ± SD
			Female	Male	Total		
1	10 × 7	6 × 2	4.750 ± 0.40	3.250 ± 0.35	8.000 ± 0.59 ^a	47.56 ^a	36.90 ± 4.84 ^a
2	10 × 7	6 × 2	2.750 ± 0.28	1.417 ± 0.26	4.167 ± 0.42 ^a	72.72 ^a	85.83 ± 5.38 ^a
5	10 × 7	6 × 2	3.583 ± 0.58	1.833 ± 0.30	5.417 ± 0.69 ^a	64.43 ^a	43.26 ± 7.14 ^a
Control		6 × 2	10.50 ± 0.48	4.750 ± 0.43	15.25 ± 0.43		6.253 ± 2.14

^a *P* value of <0.05, which was significant with respect to the recovery of worms from untreated control group.

1.3% in the generously allowed region, and only 0.7% in the disallowed region. Verify-3D showed 87.15% of residues with an average score of >0.2, while Errat, which calculates the quality of the model by analyzing nonbonded interactions between different atom types, showed a value of 63.668 for the modeled structure. These values were further increased to 87.65% and 88.693 for Verify-3D and Errat, respectively, after minimization of the model. This minimization was performed in explicit water to relax the modeled protein. The final energy-minimized model was used for further docking analyses.

In silico docking studies. Molecular docking studies of the compounds were performed to gain an insight into the probable mechanism of binding of these molecules with the enzyme. All the compounds showed an interaction with the active-site residues of the enzyme (Fig. 4 and 5). Common hydrophobic interactions were shown mainly by Leu82, Met79, Tyr216, Val275, and Ile115, where Tyr216, having a hydroxyphenyl side chain, was also found to be involved in a pi-pi stacking interaction with the aromatic structures present in the docked conformation of ligands. Moreover, some of the polar residues constituting the active site, Ser81, Ser217, Arg168, and Glu112, and the phosphate group of the DNA molecule directed toward the active site (DC40 residue) (Fig. 5 and 6) can facilitate electrostatic interactions with the ligands bound in the active site of protein. The docking energies of all the compounds are given in Table 2. Although the correlation between docking energy and activity (*in vitro* activity) is not quite clear from the data shown in Table 2, the most active molecule (compound 2) can evidently be recognized on the basis of docking

energy. Comparative analysis of docking poses shows that flexibility in the middle part of the backbone enhances the activity (compound 2), while the presence of a cyclopropane group might restrict the rotation of the backbone. In the presence of an attached cyclopropane ring on both sides, the size of the central ring also influences the activity profile, as evident from comparisons between docking poses of compounds 3 and 6 and between those of compounds 4 and 7, where the increase in the size of the central ring (cyclopentanone to cyclohexanone) causes a shift in the position of the compound inside the active site. Moreover, the presence of halogens in the molecule (compound 5) may enhance the activity by electrostatic interactions with polar active-site residues.

Dispiro-cycloalkanones adversely affect adult worms and MF *in vitro*. All the compounds (compounds 1 to 7) had adverse effects on the motility and viability of female adult worms *in vitro*. Compound 2 was most active (LC₁₀₀, 5 μM; IC₅₀, 1.02 μM), followed by compound 5 (LC₁₀₀, 10 μM; IC₅₀, 2.3 μM) and compound 1 (LC₁₀₀, 10 μM; IC₅₀, 2.9 μM). Compound 4 had an LC₁₀₀ of 20 μM and an IC₅₀ of 5.8 μM. Compounds 3 and 6 had similar antifilarial activities, with compound 3 having an LC₁₀₀ of 40 μM and an IC₅₀ of 17.7 μM and compound 6 having an LC₁₀₀ of 40 μM and an IC₅₀ of 15 μM. Compound 7 was the least effective, with an LC₁₀₀ of 75 μM and an IC₅₀ of 35.4 μM (Tables 3 and 4). Besides affecting adult worms, all the compounds also had various degrees of adverse effects on MF. The MTT reduction assay also showed a reduction in the viability of the worms with increasing concentrations of dispiro-cycloalkanones. All the compounds were found to be safe and had SI values of >10.

***In vivo* antifilarial activity of the wBm-LigA inhibitor in a primary jird screen.** Compounds 1, 2, and 5, with promising adulticidal activity *in vitro*, were further evaluated *in vivo* in a primary screening model using jirds that were inoculated intraperitoneally with adult *B. malayi* worms. Compounds 1, 2, and 5 brought about 47.56%, 72.72%, and 64.43% reductions in recovery of *B. malayi* adult worms, respectively, over controls (*P* < 0.05). Female worm sterilization rates for compounds 1, 2, and 5 were 36.9% ± 4.84%, 85.83% ± 5.38%, and 43.26% ± 7.14%, respectively (*P* < 0.05) (Table 5). The density and the degree of motility of MF in the peritoneal fluid of jirds upon autopsy at the end of the observation period (day 52 [after initiation of treatment]) were observed to be reduced tremendously compared with those of the controls, demonstrating the adverse effect of these inhibitors on microfilarial stages as well. Compound 1 caused a 50% to 60% reduction in MF density and a 30% to 60% reduction in MF motility, compound 2 produced an 80% to 90% reduction in MF density and 70% to 90% reduced motility, and compound 5 led to a 50% to 80% reduction in MF density and 50% to 80% reduced motility, compared to those of controls.

TABLE 6 Quantification of *Wolbachia* loads in adult worms by real-time PCR

Compound	C _T value	Mean C _T value	Log copy no. of <i>ftsZ</i>	ΔC _T value
1	25.67 25.78 25.96	25.80	3.88 ^a	12.56 ^a
2	33.31 33.05 33.15	33.17	1.35 ^a	19.93 ^a
5	30.19 30.45 30.61	30.41	2.3 ^a	17.17 ^a
Control	13.23 13.10 13.39	13.24	8.2	

^a *P* value of <0.05, which was significant with respect to the control group.

TABLE 7 *In vivo* antifilarial activities of dispiro-cycloalkanones in adult *Brugia malayi* worms in *Mastomys coucha* rodents

Compound	Dose (mg/kg) × days	No. of animals × no. of experiments	Avg no. of worms recovered ± SD			% reduction in adult worm recovery over control	Mean % sterilization ± SD
			Female	Male	Total		
1	10 × 7	5 × 2	6.9 ± 1.00	5.00 ± 0.67	11.9 ± 1.3 ^a	51.02 ^a	22.34 ± 2.36 ^a
2	10 × 7	5 × 2	5.0 ± 0.68	3.9 ± 0.6	8.9 ± 0.98 ^a	63.4 ^a	72.75 ± 3.69 ^a
5	10 × 7	5 × 2	5.1 ± 0.72	4.0 ± 0.56	9.1 ± 1.22 ^a	57.96 ^a	25.43 ± 5.02 ^a
Control		5 × 2	14.10 ± 1.	10.2 ± 1.29	24.30 ± 1.97		1.27 ± 0.9

^a *P* value of <0.05, which was significant with respect to the control group.

Real-time PCR reveals a decreased wolbachial load after treatment of jirds with dispiro-cycloalkanones. The results of real-time PCR are represented in terms of C_T values. The ΔC_T value is exponentially related to wolbachial content. The results demonstrated that female worms recovered from animals treated with compounds 1, 2, and 5 exhibited 2^{13} , 2^{20} , and 2^{17} fewer *Wolbachia* bacteria than did female worms recovered from the controls ($P < 0.05$). Estimation of the copy number of the *ftsZ* gene in adult worms also clearly demonstrated a significant difference in the copy numbers of *ftsZ* between the treated and control worms ($P < 0.05$) (Table 6).

***In vivo* antifilarial activity of wBm-LigA inhibitors in a secondary mastomys screen.** Evaluation of the antifilarial efficacy of compounds 1, 2, and 5 was undertaken with a mastomys model (where the route of infection mimics that of a natural human LF infection). Compound 2 exerted 63.4% adulticidal activity and $72.75\% \pm 3.69\%$ female worm sterilization ($P < 0.05$), while compounds 1 and 5 showed comparatively less activity against adult worms, with 51.02% and 57.96% reductions in adult worm recovery and $22.34\% \pm 2.36\%$ and $25.43\% \pm 5.02\%$ female worm sterilization, respectively ($P < 0.05$) (Table 7).

Microfilarial densities in the tail blood of treated mastomys remained low in comparison to those in untreated control animals, with compound 2 showing the maximum decrease in microfilarial density throughout the observation period. However, in animals treated with compounds 1 and 5, the MF density was initially decreased up to day 8 with respect to day 0, and afterwards, there was an increase in MF density (Fig. 7).

DISCUSSION

NAD⁺-dependent DNA ligase has been considered to be a potent antibacterial target on the basis of its specific cofactor requirement, the major sequence discrepancies between prokaryotic and eukaryotic representatives, and its indispensable role in DNA metabolism. In the present investigation, dispiro-cycloalkanone compounds were explored for their inhibitory potential against the NAD⁺-dependent DNA ligase of the *Wolbachia* endosymbiont of the filarial parasite *B. malayi* (wBm-LigA). These compounds exhibited inhibition of the nick-closing and cohesive-end ligation activities of wBm-LigA in *in vitro* enzymatic assays. The mechanism of inhibition was competitive with the NAD⁺ cofactor, which indicates that the binding site of these compounds partially or fully overlaps the binding site of the NAD⁺ cofactor. This was further affirmed by *in silico* molecular docking analyses, where the inhibitors were found to interact with the active site of the enzyme through hydrophobic as well as electrophilic interactions. Thus, the dispiro-cycloalkanone series of compounds inhibits the first step of the ligase reaction, i.e., adenylation. Compound 2 was found to be the most active inhibitor in the *in vitro* enzymatic assays. The K_i value (35 nM) for compound 2 was much lower than the K_m value for NAD⁺, indicating a greater affinity of wBm-LigA for the inhibitor, which was also reflected by its lowest docking energy in *in silico* docking studies. This could possibly be one of the reasons why the enzyme inhibited wBm-LigA activity in the low-micromolar range. Furthermore, these compounds did not inhibit the activity of ATP-dependent human or T4 DNA

FIG 7 Graph showing the percent change in the MF load along the y axis for the control, compound 1, compound 2, and compound 5, with days along the x axis.

ligase at concentrations of up to 100 μM , showing the specificity of these compounds for wBm-LigA. Thus, these findings clearly supported that the dispiro-cycloalkanones were potent inhibitors of wBm-LigA.

Having established that the dispiro-cycloalkanones were potent inhibitors of wBm-LigA, the inhibitory effect of dispiro-cycloalkanones on wBm-LigA in the endosymbiont *Wolbachia* was observed. Since *Wolbachia* bacteria reside within the filarial parasite intracellularly, and it is difficult to culture them *in vitro*, the effect of dispiro-cycloalkanones on filarial parasites was observed, keeping in view the symbiotic relationship of the filarial parasite with the endosymbiont. Interestingly, all seven compounds adversely affected the motility of adult female *B. malayi* worms *in vitro* although to different degrees. Compound 2 was most active in *in vitro* enzymatic assays (MIC of 0.05 μM) and showed maximum affinity (-10.33 kcal/mol) for the target enzyme in docking studies. It was also found to be the most active compound (LC_{100} , 5 μM ; IC_{50} , 1.02 μM) against adult parasites, followed by compound 5 (LC_{100} , 10 μM ; IC_{50} , 2.3 μM). Similarly, the least active compounds in the *in vitro* enzymatic assays and the docking study were also least active in adult worms.

The most effective dispiro-cycloalkanones exhibiting wBm-LigA inhibition and adverse effects on *B. malayi* *in vitro* were further evaluated for antifilarial activity in the transplanted jird model. Of all the compounds, compound 2 appeared to be most effective, as it killed $\sim 72\%$ of the transplanted adult worms, brought about $85.56\% \pm 6.8\%$ female worm sterilization, and also significantly reduced the MF density in the peritoneal fluid of jirds. To determine whether enzyme inhibitors adversely affected adult worms by affecting *Wolbachia*, and not by direct effect on adult worms, the *in vivo*-treated parasites were processed for real-time quantitative PCR (qPCR). As determined by real-time qPCR, the C_T values of the *Wolbachia* housekeeping gene *ftsZ* were higher in worms recovered from inhibitor-treated jirds than those in control worms, indicating a low copy number of the *ftsZ* gene and thus a low *Wolbachia* load. The qPCR results argue strongly for inhibition of wolbachial replication, as the gene copy number is reduced drastically. Thus, it could be hypothesized that the inhibition of DNA ligase could affect DNA repair mechanisms and replication, which could have resulted in impaired *Wolbachia* division and may have accelerated bacterial death, which resulted in a low wolbachial load. Alternatively, DNA repair dysfunction may lead to a change in the DNA sequence affecting transcription and subsequent protein synthesis, which may result in the reduced motility, viability, and survival of the filarial parasite host. *In vivo*, the adverse effect on adult worms in rodent hosts may be attributed to the altered immune response following *Wolbachia* depletion. As in *Onchocerca volvulus*, decreased recruitment of neutrophils around the adult worms was noticed after a course of an antirickettsial antibiotic, doxycycline demonstrating an altered immune response as a result of *Wolbachia* depletion (33).

Following the screening of drugs by using the jird i.p. model, mastomys were next used to assess the efficacy of these compounds in an animal model that more closely mimics human infection and treatment. Similarly to jirds, in mastomys, compound 2 was the most effective macrofilaricide. It also caused the most female sterilization in both animal models. However, the antifilarial activity in mastomys was marginally lower than that observed in jirds. Mastomys are infected through the natural route of infection, in contrast to jirds, where the parasites are introduced

into the peritoneal cavity, which is an artificial site for *B. malayi*. The latter screen provides results much more rapidly and therefore is suitable for identifying active antifilarial agents. The mastomys model, on the other hand, more closely models human infection, as larvae are introduced subcutaneously, as in human infection; migrate to reach the lymphatics and various tissues; and release MF, which circulate in the peripheral blood, as in humans.

Thus, the present study clearly demonstrates that wBm-LigA could be an ideal drug target in anti-*Wolbachia* (A-WOL) drug discovery and development programs, and dispiro-cycloalkanones could be an effective lead to target the endosymbiont *Wolbachia*. Previous studies exploiting wolbachial recombinant proteins as antifilarial drug targets have been limited to *in vitro* evaluation of inhibitors on filarial worms (11–13). In the present study, *in vivo* studies were performed in experimental filarial models to demonstrate the *in vivo* effect of wBm-LigA inhibition on filarial parasite survival and these enzyme inhibition results were also correlated with the low wolbachial loads in the recovered treated worms. Optimization of potency, dose-dependent efficacy, and *in vivo* pharmacokinetic properties is required to further improve antifilarial efficacy. The chemical synthesis of more compounds of the dispiro-cycloalkanone series and chemical modifications would be worth pursuing further.

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