

Moxidectin causes adult worm mortality of human lymphatic filarial parasite *Brugia malayi* in rodent models

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Abstract: Moxidectin is a macrocyclic lactone belonging to milbemycin family closely related to ivermectin and is currently progressing towards Phase III clinical trial against human infection with the filaria *Onchocerca volvulus* (Leuckart, 1894). There is a single report on the microfilaricidal and embryostatic activity of moxidectin in case of the human lymphatic filarial parasite *Brugia malayi* (Brug, 1927) in *Mastomys coucha* (Smith) but without any adulticidal action. In the present study, the *in vitro* and *in vivo* antifilarial efficacy of moxidectin was evaluated on, *B. malayi*. *In vitro* moxidectin showed 100% reduction in adult female worm motility at 0.6 μ M concentration within 7 days with 68% inhibition in the reduction of MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide dye) (which is used to detect viability of worms). A 50% inhibitory concentration (IC₅₀) of moxidectin for adult female parasite was 0.242 μ M, for male worm 0.186 μ M and for microfilaria IC₅₀ was 0.813 μ M. In adult *B. malayi*-transplanted primary screening model (*Meriones unguiculatus* Milne-Edwards), moxidectin at a single optimal dose of 20 mg/kg by oral and subcutaneous route was found effective on both adult parasites and microfilariae. In secondary screening (*M. coucha*, subcutaneously inoculated with infective larvae), moxidectin at the same dose by subcutaneous route brought about death of 49% of adult worms besides causing sterilisation in 54% of the recovered live female worms. The treated animals exhibited a continuous and sustained reduction in peripheral blood microfilaraemia throughout the observation period of 90 days. The mechanism of action of moxidectin is suggested to be similar to avermectins. The *in silico* studies were also designed to explore the interaction of moxidectin with glutamate-gated chloride channels of *B. malayi*. The docking results revealed a close interaction of moxidectin with various GluCl ligand sites of *B. malayi*.

Keywords: Nematode, chemotherapy, macrocyclic lactone, jirds, mastomys, microfilaricidal, macrofilaricidal

Wuchereria bancrofti (Cobbold, 1877) and *Brugia malayi* (Brug, 1927) are the major nematode parasites responsible for lymphatic filarial disease (LF) in humans. LF affects people living in approximately 73 countries and leaves more than 1.4 billion people at risk and 120 million population already infected (WHO 2013). It is ranked as the second most common cause of permanent and long-term disability causing major impediment to socioeconomic development (WHO 1997). The mainstay antifilarial drugs are limited in number (diethylcarbamazine – DEC, albendazole – ALB and ivermectin – IVM), which are principally microfilaricidal with poor macrofilaricidal (adulticidal) activity.

Moxidectin (MOX) is a macrocyclic lactone structurally within the milbemycin family and derived from the actinomycete *Streptomyces cyanogriseus* Yen, 1956. Treatment of *B. malayi*-infected animals with MOX has been reported to exhibit microfilaricidal activity and altered embryo morphology; however, adulticidal effects

were limited to rodent filariid *Acanthocheilonema viteae* (Krepkogorskaya, 1933) (see Schares et al. 1994). Tompkins et al. (2010) reported the inhibition of microfilaria release by *in vitro* treated adult *B. malayi* with significantly decreased expression of *Wolbachia* surface protein (*wsp*). The effects in case of *Onchocerca volvulus* (Leuckart, 1894) included cuticular/structural damage and reduced motility of microfilariae (Tagboto and Townson 1996, Mancebo et al. 1997) apart from morphological alterations in hypodermal cells of adult males (Strote et al. 1990, Tagboto and Townson 1996). The microfilarial motility was also reduced in case of *Onchocerca lienalis* (Stiles, 1892), *Monanema martini* Bain, Bartlett et Petit, 1986 and *Litomosoides sigmodontis* (Chandler, 1931) with adverse effects on female worm embryogenesis (Tagboto and Townson 1996). MOX is being tried in patients with onchocerciasis to investigate its potency against adult worms. Past studies on MOX revealed its good safety and tolerance between 3 and 36 mg doses in

humans (Cotureau et al. 2003). The clinical trials of this drug have been launched in Africa through WHO collaborative efforts of Special Program for Research and Training in Tropical Diseases and Wyeth Pharmaceuticals, and the data have been promising so far (Chen et al. 2002, WHO 2008, Awadzi et al. 2014). The primary mode of action of MOX is similar to IVM affecting glutamate-gated chloride channels (GluCl) and thus causing paralysis and parasite death (Cully et al. 1994, Blackhall et al. 1998, Forrester et al. 2002). Some reports also demonstrate the efficacy of MOX against those nematodes that did not respond to IVM (Craig et al. 1992, Coles et al. 1994), suggesting some differences in the mode of action of these two compounds.

The proposed study has been planned to evaluate the *in vitro* and *in vivo* antifilarial efficacy of MOX against lymphatic filarial parasite, *B. malayi*, using comparatively higher doses keeping in view of its reported promising activity against *O. volvulus* and the progression of this drug towards advanced clinical phase. *In silico* studies were also performed to examine the interaction between drug and its target site GluCl.

MATERIALS AND METHODS

Ethics statement for animal study

The use of rodent animals, jirds (*Meriones unguiculatus* Milne-Edwards) and mastomys (*Mastomys coucha* Smith), and the animal handling protocols used in the study were approved by the Institutional Animal Ethics Committee (IAEC) of the Central Drug Research Institute (CDRI), Lucknow, India. Recommended guidelines given in the Guide for the Care and Use of Laboratory Animals (National Research Council 1996) were strictly followed during the study. Transplantation of adult nematodes of *Brugia malayi* in the peritoneal cavity of jird was performed under ketamine anesthesia (50 mg/kg of body weight, Aneket, Mumbai, India).

In vitro activity of MOX

Animals were kept under hygienic and standard housing conditions with 12 h light/dark cycle at $23 \pm 1^\circ\text{C}$ temperature and $55 \pm 10\%$ relative humidity in the National Laboratory Animal Centre (NLAC) of the CDRI, Lucknow, India. Animals received a standard pellet diet and water *ad libitum*.

Parasites

Adult worms and microfilariae (mf) of *B. malayi* were isolated aseptically by repeatedly washing the peritoneal cavity of donor jirds infected 4–5 months earlier by intraperitoneal inoculation of ~150 infective larvae (L3). Adult worms were washed in sterile Roswell Park Memorial Institute (RPMI)-1640 medium containing antibiotic-antimycotic mixture (Streptomycin 100 µg/ml and penicillin 100 U/ml) and transferred to the fresh medium fortified with 5% fetal bovine serum. Mf were freed of host cells after passing the peritoneal washing through 5.0 µm membrane filter (Whatman Limited, Maidstone, England) and pelleted subsequent to washing (McCall et al. 1973). All actively moving worms were placed on a 24-well culture plate (1 adult female or male/well) in the presence or absence of drugs. The medium was replaced every 48 h with the fresh me-

dium to which drugs were added. The parasites were incubated for 10 continuous days at 37°C in CO_2 (5%) incubator, whereas mf were placed in 48-well culture plates (100 live Mf/well) and incubated with drugs in the same manner.

Primary *in vitro* testing

The macrofilaricidal and microfilaricidal efficacies of MOX (Sigma-Aldrich Co., St. Louis, USA) have been evaluated *in vitro*. IVM (Sigma-Aldrich) was used as the standard drug in all the *in vitro* experiments since DEC is known to be *in vitro* inactive. Drugs were added to the culture in duplicate and experiment was done three times. The drugs were used at various concentrations (5–80 µM) using two-fold dilutions. Medium in the control wells contained DMSO as the vehicle in various quantities corresponding to each experimental well.

Motility of both the life stages of *B. malayi* was observed at every 24 h under an inverted microscope and scored as very high (4+, 0% inhibition in worm motility), moderate (3+, 1–49% inhibition), slow (2+, 50–74% inhibition), paralysed (1+, 75–99% inhibition) and completely immotile (D, 100% inhibition). The adult worms were then processed for viability assay using 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye (Mukherjee et al. 1998).

MTT reduction assay on adult parasites

The experiment was terminated on day 10 after drug exposure. The worms that became completely immotile were transferred to the fresh drug-free medium (37°C for 2 hours) to confirm the non-reversal of the motility. Treated and control adult worms were processed for the MTT reduction assay, which was carried out in 96-well culture plate containing 0.1 ml MTT (0.5 mg/ml) in phosphate buffer saline (PBS, Sigma-Aldrich) for the adult worms as described earlier (Mukherjee et al. 1998). In brief, worms were washed in PBS, suspended in MTT solution (0.5 mg/ml) and incubated for 45 min. at 37°C . Each worm was transferred to 0.1 ml of DMSO in another 96-well culture plate, which was left at 37°C for 45 min for solubilisation of formazan (reduced product of MTT). The worms were removed and the plate was gently agitated to disperse the colour evenly and the absorbance of the solubilised formazan was measured at 510 nm using multiwell plate reader (Tecan, Untersbergstrasse, Grodig, Austria) against DMSO blank. The OD value of each well was compared with the mean OD value of the corresponding control well and percent inhibition in MTT reduction by the treated parasite over that of untreated control worm was calculated.

Secondary *in vitro* screening of MOX

The adult and mf were exposed to various concentrations of each drug to evaluate IC₅₀ (50% inhibitory concentration), CC₅₀ (50% cytotoxicity) and SI (selectivity index). The drugs showing lethal effects on parasites at the lowest concentration of 5 µM were subjected to further testing at still lower concentrations of 0.15–5.0 µM in two-fold dilutions to assess IC₅₀. An Excel software-based line graphic template after plotting the concentration value of each sample against percent inhibition of motility of the parasite on the x and y axis, respectively, determined the IC₅₀ (Ramirez et al. 2007). The CC₅₀ of each drug was assessed *in vitro* on Vero monkey kidney cell line, as described earlier (Misra et al. 2011). The selectivity index (SI) was calculated as the ratio between CC₅₀ and IC₅₀. The drugs showing SI ≥ 10 were considered safe for *in vivo* follow up.

Antifilarial activity of MOX against *B. malayi* in transplanted jird model (primary screening)

Six to eight weeks old jirds were intraperitoneally transplanted with 10 female and 5 male adult worms of *B. malayi* recovered from infected jird harbouring 4–8 months old infection. The worms were introduced into the recipients' peritoneal cavity through surgical incision made in the ventrolateral abdominal wall of anaesthetised jird. On day seven a drop of peritoneal fluid was aspirated to check the presence of live microfilariae to ensure successful transplant. Jirds harbouring live mf in the peritoneal cavity were selected for treatment.

A single dose of MOX was administered subcutaneously or orally at 10, 20 and 40 mg/kg as suspension in 0.05% Tween 80 (Fig. 1). DEC (Sigma-Aldrich); the standard filaricide was also given by the two routes at 100 mg/kg.

Effect of drugs on parasitaemia

At the end of observation period (50 days), animals were euthanised for mf and adult worm recovery from the peritoneal cavity as reported earlier (Misra-Bhattacharya et al. 2004). Briefly, the peritoneal cavity was washed initially with 5 ml PBS (1 ml at a time), 10 µl of this fluid was observed under microscope to count mf. The adult worms were washed in PBS (pH 7.2), counted and observed for their motility, calcification and death or encapsulation. Surviving females from each group were teased individually in a drop of PBS and the condition of the embryonic stages in the uteri were examined microscopically and compared with those recovered from control group (Misra et al. 1984).

Antifilarial activity of MOX against *B. malayi* in L3 induced *Mastomys* model (secondary screening)

Mastomys coucha infected 5–8 months earlier by subcutaneous inoculation of ~100 L3 and showing progressive rise in microfilaraemia were selected for the treatment. Single dose of 20 mg/kg MOX was administered subcutaneously (s.c.) and micro- and macrofilaricidal efficacies were evaluated as described earlier (Misra-Bhattacharya et al. 2004). Briefly, blood smears of 10 µl tail blood were made just before the initiation of treatment (day 0) and after treatment on days 8 and 15, and thereafter at fortnightly interval till day 90. Changes in microfilarial density in comparison to pretreatment level were expressed as the percent change in microfilaraemia. At the end of the observation period (day 91), the treated and control *Mastomys* were euthanised and various tissues (lungs, heart, testes, lymph nodes) were isolated and teased gently in phosphate buffered saline (PBS, pH 7.2) to recover adult parasites (Misra-Bhattacharya et al. 2004). The recovered females were teased individually in a drop of PBS to observe embryostatic effect, if any.

Statistical analysis

Each experimental group contained five or six animals and experiment was done at least three times. The percent reduction in worm recovery was assessed by comparing the mean values of the treated group with those of the respective untreated control group. Statistical analysis was done by comparing each treated group and the respective control group using one-way analysis of variance (nonparametric) using Dunnett's test with the help of GraphPad Prism Version 5.01 (GraphPad Software, Inc.). Statistical significance was as follows: *p* values of < 0.05 significant, < 0.01 highly significant and < 0.001, very highly significant was marked as *, ** and ***, respectively.

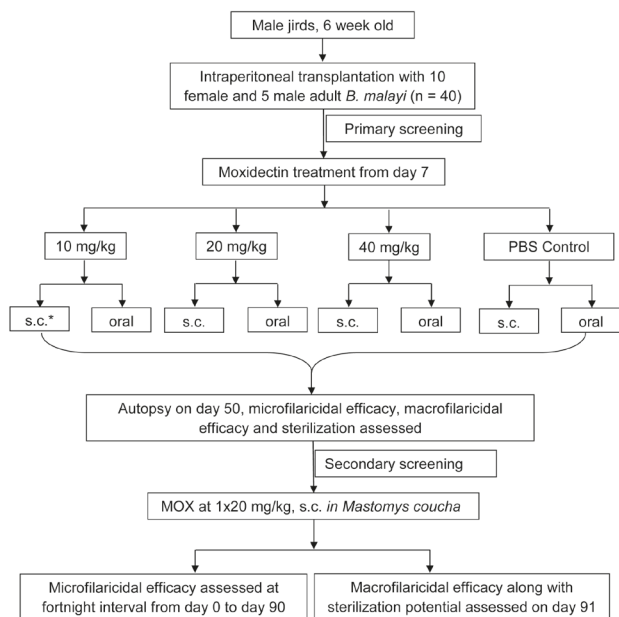


Fig. 1. Schematic representation of *in vivo* antifilarial activity of moxidectin against *Brugia malayi* in rodent models. Abbreviation: s.c. – subcutaneous.

Homology modeling of GluCl of *B. malayi*

The putative *B. malayi*–glutamate-gated chloride channel (*Bm*-GluCl) amino acid sequence was retrieved from NCBI, (GenBank Acc. No. ADR74384.1). The homology model of the *Bm*-GluCl was generated using Phyre2 server at the Imperial College, London, UK (Kelley and Sternberg 2009) using the *Caenorhabditis elegans*–GluCl structure as a template PDB id: 3RIA (Hibbs and Gouaux 2011) identified by PSI-BLAST search against proteins in the Protein Data Bank. The generated model was further refined through ModRefiner server (Xu and Zhang 2011). For evaluation and validation of the model, Ramachandran Plot was generated from PROCHECK (Laskowski et al. 1993). Knowledge-based energy curve for calculating z-score was performed by ProSA-web (Sippl 1993).

Docking study of MOX with GluCl

Two dimensional (2D) structure of MOX was retrieved from Pubchem database. The two dimensional structure was converted to 3D structure by OpenBabelGUI interface (O'Boyle et al. 2011). The 3D ligand site was utilised for predicting ligand binding sites in the *Bm*-GluCl model (Wass et al. 2010). Simplified protein-ligand docking was carried out on HEX server (<http://hexserver.loria.fr/>) (Macindoe et al. 2010). All the visualisation, 2D ligand interaction diagrams and analysis were done on Maestro 9.2 and PyMol (Schrödinger, LLC, New York, USA).

RESULTS

In vitro antifilarial activity of moxidectin on adults and microfilariae of *Brugia malayi*

The antifilarial effect of moxidectin (MOX) on adult females *Brugia malayi* at various concentrations ranging between 0.15 and 5 µM (see Table 1). MOX completely inhibited the adult female and male worm motility at

Table 1. Concentration-dependent *in vitro* antifilarial activity of moxidectin (MOX) against *Brugia malayi* adult and microfilariae.

Drugs	Concentration (µM)	Adult						Microfilariae	
		Female			Male				
		Motility score	No.of days	Percent of inhibition in MTT reduction over control	Motility score	No. of days	Percent of inhibition in MTT reduction over control	Motility score	No. of days
MOX	5.0	D	2	65.87	D	5	47.83	D	7
	2.5	D	3	54.98	D	6	49.74	D	8
	1.25	D	7	64.93	D	9	50.84	1+	10
	0.6	D	7	67.67	1+	10	39.72	2+	10
	0.3	1+	10	34.34	2+	10	31.63	2+	10
	0.15	3+	10	21.76	2+	10	28.58	4+	10
IVM	5.0	D	3	19.90	D	4	31.23	D	7
	2.5	D	3	23.04	D	6	27.84	D	8
	1.25	D	9	45.50	D	9	39.02	1+	9
	0.6	D	9	18.35	1+	10	24.43	2+	10
	0.3	2+	10	16.94	2+	10	18.18	4+	10
	0.15	3+	10	25.36	2+	10	15.94	4+	10
Control	5.0	4+	10	-	4+	10	-	4+	10
	2.5	4+	10	-	4+	10	-	4+	10
	1.25	4+	10	-	4+	10	-	4+	10
	0.6	4+	10	-	4+	10	-	4+	10
	0.3	4+	10	-	4+	10	-	4+	10
	0.15	4+	10	-	4+	10	-	4+	10

D – dead; IVM – ivermectin; MTT – 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide dye.

Table 2. *In vitro* antifilarial activity of moxidectin (MOX) against *Brugia malayi* adult and microfilariae.

Drugs	Adult								Microfilariae			
	Female				Male							
	MIC ^a (µM)	IC50 ^b (µM)	CC50 ^c (µM)	SI ^d	MIC ^a (µM)	IC50 ^b (µM)	CC50 ^c (µM)	SI ^d	MIC ^a (µM)	IC50 ^b (µM)	CC50 ^c (µM)	SI ^d
MOX	0.60	0.24	22.6	93.5	1.25	0.19	22.6	121.7	2.5	0.81	22.6	27.8
IVM	0.60	0.30	53.2	177.2	1.25	0.19	53.2	285.8	2.5	1.08	53.2	49.4

^a the MIC (minimum inhibitory concentration) of adult *Brugia malayi* worms and mf were determined as the minimum drug concentration causing total irreversible inhibition in worm immobility (death); ^b the IC50 (50% inhibitory concentration) were determined by use of an Excel software-based line graphic template after plotting the concentrations of each sample against percent inhibition of parasite motility on the x and y axes, respectively; ^c the CC50 (50% cytotoxic concentration) of each inhibitor was determined on Vero cells; ^d SI (selectivity index) = CC50/IC50.

concentration of 5 µM within 2 and 5 days, respectively, while motility of microfilariae (mf) completely stopped within 7 days at this concentration (Table 1). At lower concentrations (0.15–5.0 µM) for IC50 evaluation, MOX caused complete worm immobility up to 0.6 µM in 7 days exposure time with significant inhibition in MTT reduction (68%; see Table 2). Minimum inhibitory concentration (MIC) of MOX against male adult worm was found to be 1.25 µM on drug exposure for 9 days. The IC50 values of MOX against adult female and male worm was evaluated as 0.24 µM and 0.19 µM, whereas IVM exhibited 0.30 µM and 0.19 µM IC50 on the two sexes, respectively (Table 2). In case of mf, the IC50 of MOX was found to be 0.81 µM, which is lower than that of the standard drug ivermectin (1.08 µM) (Table 2). MOX displayed 23 µM CC50 when tested on Vero monkey kidney cell line. On calculating the SI values, MOX exhibited good safety index with SI values 94 (adult female) and

122 (male), respectively (Table 2). In contrast, SI value of MOX for mf was 27.8 (Table 2). Based on these safety data, *in vivo* screening of these two drugs were taken up.

***In vivo* evaluation of antifilarial activity of MOX on *B. malayi* in jirds (primary screening)**

Macrofilaricidal activity

The macrofilaricidal activity of MOX at 10, 20 and 40 mg/kg was found to be 49% ($p < 0.001$), 63% ($p < 0.001$) and 66% ($p < 0.001$) via oral route, whereas the macrofilaricidal efficacy was improved using subcutaneous administration, with the corresponding values of 56% ($p < 0.001$), 73% ($p < 0.001$) and 71% ($p < 0.001$), respectively (Fig. 2). DEC when given subcutaneously for five consecutive days at 100 mg/kg exhibited 41% ($p < 0.001$) reduction in worm recovery, whereas the oral dose caused negligible (13%) worm mortality.

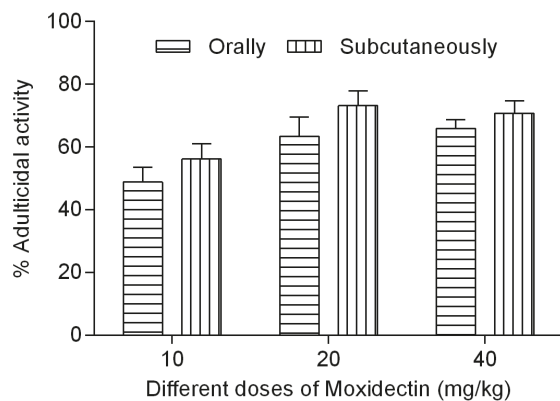


Fig. 2. Comparative adulticidal efficacy of different treatment regimens of moxidectin in intraperitoneally adults of *Brugia malayi* transplanted jird model both by oral or subcutaneous routes. Adulticidal activity is shown in terms of % reduction in adult worm recovery.

The optimal effective dose (20 mg/kg, subcutaneously (s.c.) \times 1 day) was then followed in the secondary *Mastomys* screening.

Determination of sterilisation potential

Adult female parasites recovered from the peritoneal cavities of the transplanted treated jirds were teased to assess the effect of drugs on intrauterine development. At all the three doses, MOX-treated females showed degeneration of partial population of eggs and embryos (Fig. 3). Around 60–70% of females had such deformed eggs and embryos in their uteri.

Microfilaricidal activity

Microfilarial density in MOX-treated jirds at 10, 20 and 40 mg/kg demonstrated significant reduction (51%, $p < 0.001$; 60%, $p < 0.001$, and 63%, $p < 0.001$, respectively) on oral administration, while 54% ($p < 0.001$), 63% ($p < 0.001$) and 66% ($p < 0.001$) via s.c. route, respectively. Approximately 15% of the peritoneal mf were dead in all the three groups. In addition to their numbers, the degree of motility of mf at the end of observation period (day 50) was also reduced as compared to that of controls demonstrating adverse effect of MOX on microfilarial stages. In contrast, DEC brought about 25% reductions in microfilaraemia.

In vivo evaluation of antifilarial activity of MOX on *B. malayi* in *Mastomys coucha*

Adulticidal activity

In vivo antifilarial efficacy of MOX at 20 mg/kg s.c. \times 1 day was found to be 49% ($p < 0.001$) with 54% ($p < 0.001$) (Fig. 4) adverse effect on female embryogenesis in *Mastomys coucha* model. DEC, which was given orally for five consecutive days at 50 mg/kg, exhibited 40% ($p < 0.01$) reduction in adult worm recovery.

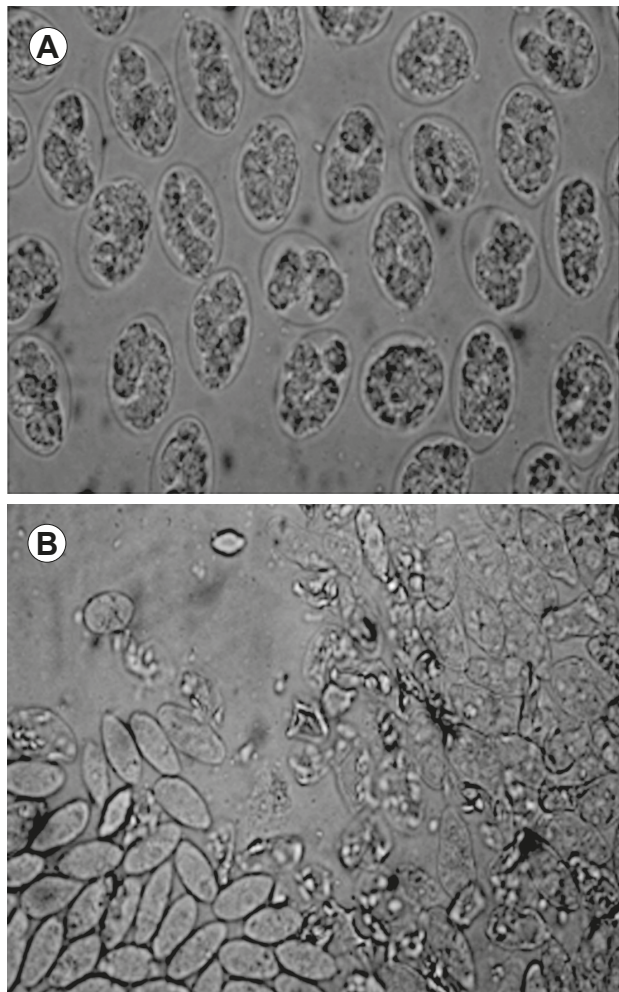


Fig. 3. Adult female of *Brugia malayi* isolated from peritoneal cavity of control and moxidectin treated jirds. Moxidectin treatment leads to deformed and degenerated eggs (Fig. 3B) compared to control worm (Fig. 3A).

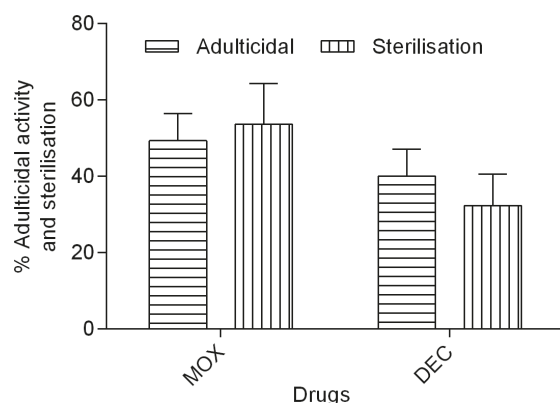


Fig. 4. Adulticidal and sterilisation activity of moxidectin (MOX) at 20 mg/kg s.c. \times 1 day in *Mastomys coucha*. The antifilarial activity is shown as % reduction in adult worm recovery compared to untreated control. Moxidectin demonstrated mortality of 49% of adult worms and 54% of recovered females were sterilised, which is markedly higher than standard filaricide diethylcarbamazine (DEC) treatment.

Table 3. Change (mean \pm standard error of mean) in microfilarial density per 10 μ l of tail blood during observation period, post treatment (%).

Drugs	% change microfilariae/10 μ l tail blood post treatment –on days						
	7	15	30	45	60	75	90
MOX (20 mg/kg)	-17.6* \pm 14.5	-21.4 \pm 7.0	-35.1 \pm 18.3	-66.9 \pm 17.5	-68.8 \pm 10.9	-64.0 \pm 15.2	-70.6 \pm 12.4
DEC(50 mg/kg)	-70.4 \pm 2.4	-60.4 \pm 6.3	-38.9 \pm 7.9	-15.4 \pm 2.6	31.2 \pm 46.3	79.95 \pm 47.94	116.8 \pm 61.4
Control	+92.9 \pm 3.9	+146.4 \pm 15.5	+176.8 \pm 24.1	+298.8 \pm 14.4	+418.9 \pm 30.1	+515.9 \pm 59.5	+587.7 \pm 55.8

*p < 0.05 low significant

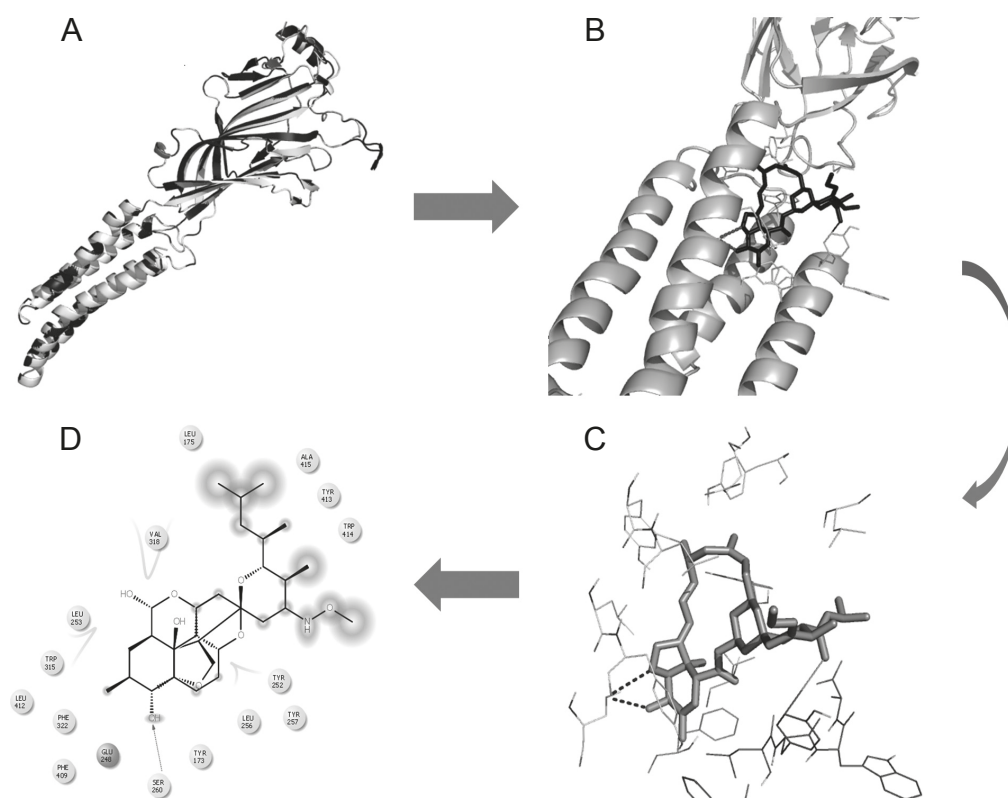


Fig. 5. Superimposed 3D homology model of *Brugia malayi* - glutamate-gated chloride channel *Bm-GluCl* (Fig. 5A in black) and the *Caenorhabditis elegans* GluCl (Fig. 5A in gray). Molecular docking was performed to examine the binding profile of moxidectin with the putative *Bm-GluCl* (Fig. 5B,C). 2D ligand interaction diagram for moxidectin and *Bm-GluCl* (Fig. 5D) clearly shows LEU253, TRP315, LEU412, PHE322, PHE409, GLU248, TYR173, LEU256, TYR257, TYR252, TRP414, TYR413, ALA415, LEU175 and VAL318 amino acid residues that are in close proximity while SER260 binding is same as in ivermectin *C. elegans* GluCl interaction.

Microfilaricidal (MIF) activity

A single dose of MOX at 20 mg/kg s.c. was capable of reducing microfilaraemia. Mf level started decreasing gradually from day 7 post treatment and the reduction endured till the observation period of 90 days reaching 71% ($p < 0.001$) from the pretreatment level (Table 3). DEC caused drastic reduction in microfilaraemia on day 7 that continued up to day 45 and subsequently mf density started increasing (Table 3).

Homology modeling of *Bm-GluCl* and docking with MOX

Bm-GluCl shares 81% sequence identity with over all 65% predicted structure similarity with the template

from *Caenorhabditis elegans* (Maupas, 1900) (PDB id: 3RIA), which is GluCl in complex with Fab and ivermectin (Fig. 5A). The homology model generated from Phyre2 was further refined to make it the most suitable for the docking studies. Ramachandran plot testified that 90% of the residues were in the most favoured regions and 8.1%, 1.6% and 0.8% of the residues rested in an additional allowed region, a generously allowed region, and a disallowed region, respectively, thereby indicating that the model was geometrically acceptable (Fig. 6A). The z-score for the homology model fits well with the experimentally determined X-Ray crystal structure of GluCl of *C. elegans* (Fig. 6B).

The analysis of binding sites was performed based on the published crystal structure of GluCl (Hibbs and

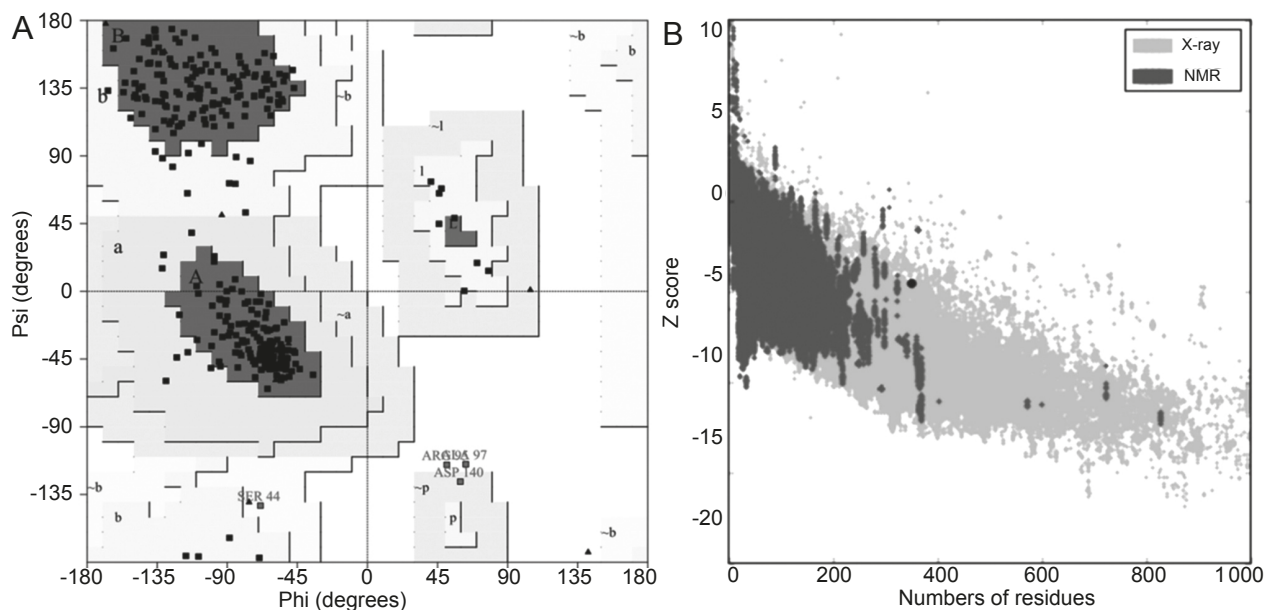


Fig. 6. Quality and assessment of the *Brugia malayi*-glutamate-gated chloride channel (*Bm-GluCl*) homology model. **A** – Ramachandran plot from the PROCHECK server revealed the acceptable geometry of the homology model; **B** – the z-score for *Bm-GluCl* homology model. The score (-5.25) generated through ProSA-web server is within the range of experimentally similar X-ray solved *Caenorhabditis elegans* GluCl protein structure (NMR – Nuclear Magnetic Resonance).

Gouaux 2011). Automated blind docking was performed to examine the binding/interaction of MOX with the putative *Bm-GluCl*, which is quite similar to ivermectin (Fig. 5B,C). 2D ligand interaction diagram for moxidectin *Bm-GluCl* clearly shows that LEU253, TRP315, LEU412, PHE322, PHE409, GLU248, TYR173, LEU256, TYR257, TYR252, TRP414, TYR413, ALA415, LEU175 and VAL318 amino acid residues are in close proximity as compared to ivermectin, while SER260 shows similarity with ivermectin and *C. elegans* GluCl interaction (Fig. 5D) (Hibbs and Gouaux 2011).

DISCUSSION

The MDA programs for elimination and control of onchocerciasis and LF (Ottesen et al. 2008) demonstrate positive results in terms of decreased microfilarial levels, reduced morbidity and interruption of disease transmission. However, concerns are being raised about the feasibility of filarial elimination in absence of a promising macrofilaricide. The indications of emergence of resistance to IVM, which constitutes the mainstay of both MDA programs (Schwab et al. 2005, Osei-Atweneboana et al. 2007), are also worrisome. A large chunk of global population at the risk of LF infection cannot solely rely on the discovery of a new chemical entity, which takes several years to become a drug. MOX, a well known veterinary parasitocidal drug, has been shown to have promising macrofilaricidal activity in animal models of onchocerciasis and presently this drug is undergoing larger phase III studies.

The present study therefore aims at filling the lacuna of macrofilaricidal drug by evaluating the *in vitro* and *in vivo* antifilarial activity of MOX against experimental human lymphatic filarial parasite *Brugia malayi* maintained in rodent host. Tompkins et al. (2010) compared the *in vitro* activity of MOX with IVM on *B. malayi* and reported that both these drugs inhibit the release of microfilariae by gravid female worms within four days. They also reported a significant decrease in wsp expression after *in vitro* ivermectin or moxidectin exposure suggesting some effect on bacteriae of the genus *Wolbachia* (Hertig, 1924). In the current study, we attempted to evaluate IC₅₀ and selectivity index (SI) of both these drugs on *B. malayi*. A significant reduction in the motility of two sexes of worms was observed as also reported by Tompkins group. However, the lethal effect of MOX in our study was more conspicuous on female *B. malayi* rather than males in contrast to their findings. The females had adverse effects on the embryonic development that could be the reason for early lethality on female worms (Strote et al. 1990, Schares et al. 1994, Tagboto and Townson 1996, Breton et al. 1997). MTT reduction, a reliable method for testing worm viability (Sylvester 2011) indicated that MOX not only paralysed the worms but also had deleterious effect on its viability (Mukherjee et al. 1997, 1998). MOX did not exhibit lethal effect on the microfilariae either kept in cultures or those released *in vitro* by female parasites substantiating the effect of drug on neurons leading to worm paralysis thereby impairing the microfilaria ejection capability of females. The selectivity index (SI) of MOX was high and thus considered safe for further *in vivo* follow up.

Macrocyclic lactones (ML) are highly effective anthelmintics giving long protective period after administration because of their extensive distribution into fat. The pk parameters of MOX were earlier determined in sheep after single s.c. or oral drench dose of 0.2 mg/kg. MOX was detected in the plasma at the first sampling time (1 h) and up to at least 60 days with the same bioavailability using both the routes (Alvinerie et al. 1998). In pigs, MOX and IVM were administered by an intravenous injection at a dose of 300 µg/kg, and the plasma kinetics were very different, MOX having a greater apparent volume of distribution, longer distribution and longer detection (> 40 days) than IVM (8–10 days) (Craven et al. 2001). In another study undertaken in humans, the C_{max} and AUC observed following administration of the liquid formulation of MOX were 28.6% and 28.8% higher, respectively, and t_{max} 0.9 h shorter compared with tablets (Korth-Bradley et al. 2013).

In primary jird screen, MOX was administered at the doses higher than those earlier reported to be ineffective such as 5 mg/kg (Schaes et al. 1994). At 10, 20 and 40 mg/kg in a single dose by both s.c. and oral route, MOX revealed moderate to high degree of mortality of adult parasites (49–73%) in the current investigation. A single dose of MOX was used in the current investigation based on the earlier report by Schaes et al. (1994) and keeping in mind the above discussed pharmacokinetics of MOX, which shows high plasma concentration for quite a long period. There was not much difference in antifilarial activity at 20 and 40 mg/kg doses by two routes of administration, however, the efficacy was marginally superior at all the three doses by subcutaneous route as also reported earlier in case of sheep parasite (Alvinerie et al. 1998).

The decrease in Mf density in the peritoneal fluid may be correlated with the decreased release of microfilariae by female worms as observed *in vitro* and may be attributed to an inefficient nervous system and/or insemination. *Mastomys coucha* infected subcutaneously with *B. malayi* infective larvae serves as a secondary screening model being more akin to human filarial infection because of natural route of infection unlike the primary screening model jirds which receives adult parasites surgically into the peritoneal cavity. On further evaluation in the *Mastomys* model, MOX caused mortality of around 50% of adult parasites and sterilised 54% of the surviving female worms at a single subcutaneous dose of 20 mg/kg. The standard filaricide DEC exerted 40% adulticidal action with moderate female sterilisation at a much higher dose of 50 mg/kg. In *Mastomys*, the adulticidal activity was lower than that observed in jird at the same dose level. It is quite possible that dose higher than 20 mg/kg might have shown better results. MOX brought about a sustained and continuous decrease in microfilarial density in

the blood of *Mastomys* after treatment indicating a possible adulticidal or embryostatic action.

IVM is used in combination with ALB for LF control programs in Africa. Our earlier work on IVM against *B. malayi* in *M. coucha* at 2 mg/kg s.c. in a single dose indicated slow and ~56% (post 60 day of treatment) reduction in mf density, which could not be retained at the same level till day 105 post treatment (Bajpai et al. 2007). IVM also did not demonstrate any significant macrofilaricidal activity. In contrast, MOX was highly effective and produced a slow but progressive decrease in mf density, which reached 80% decline till the end of observation period of 90 days. The adulticidal action was around 50% in contrast to 25% shown by ivermectin. However, IVM had better embryostatic action.

It is now well accepted that IVM acts on the GluCl channels, but interaction of MOX with these channels is still controversial (Prichard et al. 2012). MOX structure shares a 16-membered macrocyclic unit with IVM, but IVM possesses a disaccharide substitute at C-13 position, which is not present in MOX and it is rather substituted at C-23 and C-25 positions (Shoop et al. 1995). MOX was also effective in treating nematode infection when IVM showed failure. All these findings indicate some differences in the mode of action of these two drugs (Craig et al. 1992, Coles et al. 1994). Martin (1996) assumed that the two drugs share a common mechanism of action. Nevertheless, cross-resistance between the two drugs has also been reported (Conder et al. 1993).

There are inherent differences in the GluCl of different nematode species and therefore concentration of same drug varies in various nematode species to activate the channels (Behnke et al. 1993, Sheriff et al. 2002, Holden-Dye and Walker 2006). To investigate this further, we applied a molecular modeling approach and results suggest that the GluCl of *B. malayi* have the same basic fold as other members of the Cys-loop ligand-gated ion channels. Docking of MOX was performed with GluCl of *B. malayi* to check *in silico* interaction of this drug with ligand channels and a closer proximity was visualised between various ligand sites of GluCl and MOX suggesting a high probability of antifilarial activity of MOX against human lymphatic filarial parasite *B. malayi*.

To summarise, MOX, a well known drug of veterinary use and presently in advanced stage of clinical trials against onchocerciasis showed adulticidal activity against human lymphatic filarial parasite *B. malayi* in rodent models at 20 mg/kg by subcutaneous or oral route. The drug targeting approaches can minimise the dose and administration schedule and the findings reveal that MOX has a bright prospect as an antifilarial drug against lymphatic filarial parasites. Thus MOX may also be tried in combination with ALB to control transmission of LF with added advantage of adulticidal action.

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