

COMPARATIVE ASSESSMENT OF THE *IN VITRO* SENSITIVITY OF *BRUGIA MALAYI* INFECTIVE LARVAE TO ALBENDAZOLE, DIETHYLCARBAMAZINE AND IVERMECTIN ALONE AND IN COMBINATION

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Abstract. The antifilaricidal drugs ivermectin (IVM), diethylcarbamazine (DEC), and albendazole (ALB), used alone or in combinations against infective third-stage larvae (L3) of nocturnally subperiodic (NSP) *Brugia malayi* (Narathiwat strain), were tested *in vitro* for sensitivity, for 7 days. IVM alone reduced larval motility at concentrations of 10^{-7} , 10^{-6} , and 10^{-5} M on day 3. DEC alone also had this effect at concentrations of 10^{-6} , 10^{-5} , and 10^{-4} M on day 2. ALB alone did not have this effect throughout the experiment, at various concentrations. However, it had greater effect when used in combination with either DEC or IVM. The result also indicated that DEC or IVM, when used in combination with ALB at concentrations of 10^{-6} M/ 10^{-6} M, and 10^{-5} M/ 10^{-5} M was effectively better than each drug used alone at those concentrations. When both drug combinations were compared, ALB/DEC seemed to be more effective than ALB/IVM at a concentration of 10^{-6} M/ 10^{-6} M on day 3. Although IVM and DEC can reduce larval motility when used alone or in combination with ALB, they cannot kill these larvae in an *in vitro* cultivation, even at a high concentration (10^{-5} M).

INTRODUCTION

Filariasis is still a major public health problem worldwide, particularly in tropical and sub-tropical regions (WHO, 1992). In Thailand, at least two endemic areas of filariasis have been reported, *ie* Malayan filariasis due to *Brugia malayi* in the south, and Bancroftian filariasis due to *Wuchereria bancrofti* in a limited area in the west (Harinasuta *et al*, 1970; Guptavanij *et al*, 1977; Division of Filariasis, 1995). Even though a control program for Malayan filariasis has been established in Thailand for a long time, the disease is still endemic year by year.

Chemotherapy is one of the strategic tools used for reducing microfilaremia in humans. However, it is currently known that no short chemotherapeutic treatment will clear all adult parasites (McReynolds *et al*, 1993). Recently, several studies have shown the efficacy and safety of a single dose of filaricidal drug combinations, ALB+IVM or ALB+DEC (Dunyo *et al*, 2000; Horton *et al*, 2000; Ismail *et al*, 1998, 2001; Shenoy *et al*, 1999, 2000, 2002; Supali *et al*, 2002). The effective single dose of ALB (400 mg), given in combination with either DEC (6 mg/kg) or IVM (200

µg/kg), is now recommended to interrupt the transmission of lymphatic filariasis (WHO, 1994; Ottesen *et al*, 1997). Although several studies on the effect of filaricidal drugs against both microfilariae (Shenoy *et al*, 1999; 2000; 2002) and adult worms (Noroës *et al*, 1997; Gayral *et al*, 1989; Figueredo-Silva *et al*, 1996; Dreyer *et al*, 1998) have been carried out, few studies have reported the effect of these drugs against infective larvae, which are transmitted to humans by mosquitos (Deverre *et al*, 1989; Gayral *et al*, 1989).

Advanced and modernized studies of filariasis due to *B. malayi*, particularly the nocturnally subperiodic (NSP) strain from southern Thailand, are still hampered by the lack of simple, practical systems. The most successful system has used Mongolian jirds, which was a host model for NSP *B. malayi* (Narathiwat strain) (Choochote *et al*, 1986; 1991). However, an *in vitro* cultivation system and drug sensitivity test should be developed to achieve a practical and initial screening system in which to carry out a variety of investigations, *ie* biochemistry, pharmacology, physiology, immunology, and molecular biology. In addition, it is important in the study of a possible site for filaricidal drugs to kill the filarial larvae *in vitro*, where all direct response and symptomatological and specific organs can be observed. This was the first study assessing the *in vitro* drug sensitivity test against infective third-stage larvae of NSP *B. malayi* (Narathiwat strain). In

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this study, we focused on the direct effect of filaricidal drugs alone; ALB, DEC, and IVM, and their combinations (ALB+IVM and ALB+DEC) against the larvae of this parasite. The morphological changes in the treated larvae were observed under a dissecting microscope. All information from this study will contribute to important basic and advanced knowledge.

MATERIALS AND METHODS

Preparation of NSP *B. malayi* infective third-stage larvae

Infective third stage-larvae (L3) of NSP *B. malayi* (Narathiwat strain) were obtained from laboratory-reared *Aedes togoi*, which had been fed on microfilaremic cats 12 days previously. This mosquito strain was proved highly susceptible to NSP *B. malayi* (Choochote *et al*, 1987). The whole bodies of 12-day post-infection mosquitos were dissected using the aseptic technique (Suwan *et al*, 1993). Mass dissection was carried out in Hank's Balanced Salt Solution (HBSS) with antimicrobial agents (10,000 units/ml of penicillin G solution, 10,000 µg/ml of streptomycin disulfate, and 25 µg/ml of amphotericin B) in a sterile Petri dish (30 mm diameter).

Preparation of culture medium and filaricidal drugs

The culture medium used to support the growth and development of the larvae consisted of a 1:1 (v/v) mixture of NCTC-135 (Sigma) and Iscove's Modified Dulbecco's Medium (IMDM; Sigma) supplemented with 20% heat-inactivated fetal bovine serum (NI-FBS medium), a mixture of antimicrobial agents (10,000 units/ml of penicillin G solution, 10,000 µg/ml of streptomycin disulfate, and 25 µg/ml of amphotericin B) and 25 mM of HEPES (Sigma).

Preparation of filaricidal drugs

Diethylcarbamazine [N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid] citrate salt (DEC; Sigma) was dissolved in distilled water, which was diluted with culture media at various concentrations (10^{-3} M to 10^{-11} M). Ivermectin B_{1a} [N,N-Diethyl-4-methyl-1-piperazinecarboxamide] (IVM; Sigma) and albendazole [Methyl-5-(propylthio)-2-benzimidazolecarbamate] (ALB; Sigma) were dissolved in absolute methanol, which was diluted with culture media at appropriate concentrations (10^{-3} M to 10^{-11} M).

In vitro sensitivity test

The method for *in vitro* cultivation mainly followed the method described by Tippawangkosol *et al* (2002). Fresh L3 was washed by repeatedly transferring to fresh media with a mixture of antimicrobial agents in

culture dishes. Thirty L3 were pipetted into each well of a 24-well culture dish, which contained 0.9 ml of culture media (NI-FBS) and 0.1 ml of filaricidal drugs alone, *eg* ALB, IVM, and DEC and their combinations (ALB/IVM and ALB/DEC) at various concentrations (10^{-3} M to 10^{-11} M). Each experiment was performed in four replicates. The culture dishes were placed in a candle jar and incubated at 37°C in an incubator for 7 days. The motility of the larvae was examined daily under a dissecting microscopy.

Evaluation of the result

The larvicidal activity of the test drugs was evaluated in terms of relative motility (RM) value (Kiuchi *et al*, 1987). A smaller RM value indicated stronger larvicidal activity, and when all larvae died, this value was 0. A minimal lethal concentration (MLC) was determined as the lowest concentration, giving an RM of 0 after 24 hours' incubation. The RM value was calculated using the motility score. The motility of the larvae was recorded using an arbitrary score of 3 (highly active), 2 (moderately active), 1 (less active), and 0 (immobile for at least 10 seconds). The results were expressed as the average motility score with respect to the exposure time of motile parasites in treated and untreated groups. Reduction of larval motility was used to evaluate the efficacy of DEC, IVM, and ALB when used alone and in combination against L3 of NSP *B. malayi* (Narathiwat strain) in an *in vitro* cultivation with NI-FBS medium for 7 days.

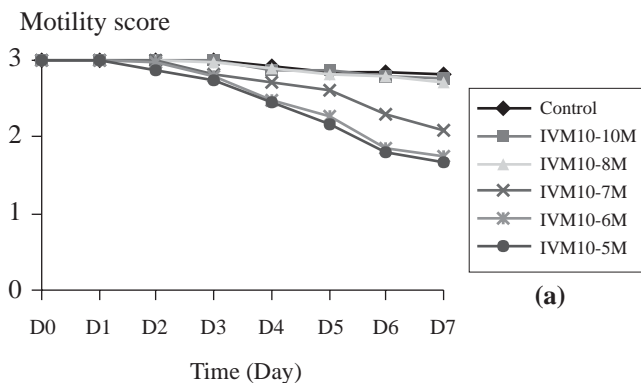
Data analysis

Comparative morphometric measurement between treated and untreated groups was evaluated by Mann-Whitney *U* test. The statistical analysis was interpreted at a p-value of 0.05.

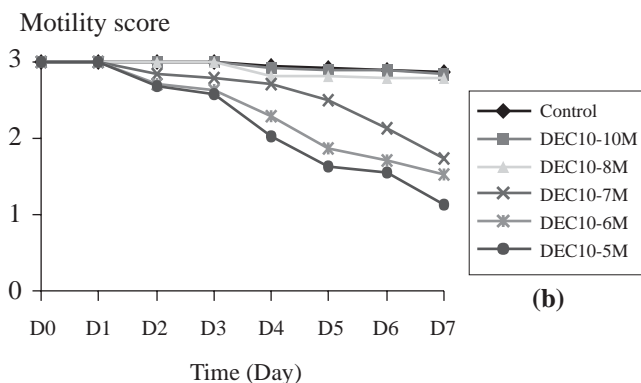
RESULTS

Efficacy of drugs alone

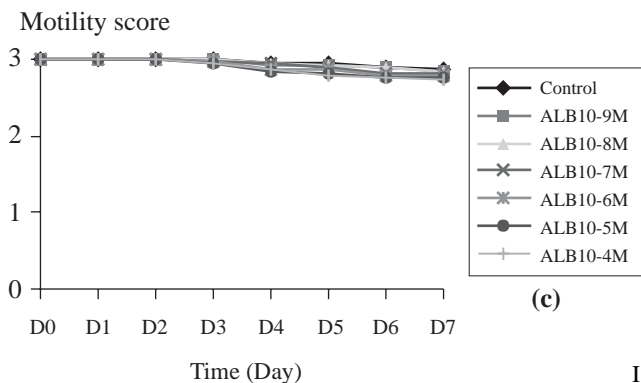
Fig 1 shows the motility of treated larvae reduced in a concentration- and time-dependent pattern after being cultured with IVM and DEC alone. None of the L3 was completely immobile after being cultured with these drugs throughout the experiment. In the control wells of all drugs, larvae showed highly active movement in the culture medium by Day 7. Both DEC and IVM alone, at concentrations of 10^{-10} M and 10^{-8} M, had no effect against larval motility. IVM alone, at concentrations of 10^{-7} M, 10^{-6} M, and 10^{-5} M, gradually reduced larval motility on Day 3, and the larvae had less active movement on Day 6 at IVM concentrations of 10^{-6} M and 10^{-5} M (Fig 1a). DEC, at concentrations of 10^{-6} M, 10^{-5} M, and 10^{-4} M, gradually reduced larval



(a)



(b)



(c)

Fig 1- Motility score of NSP *Brugia malayi* L3 after being cultured with single drugs; (a) IVM; (b) DEC; (c) ALB at various concentrations (M) in NI-FBS medium for 7 days.

motility on Day 2, and the larvae had less active movement on Day 5 at concentrations of 10^{-5} M and 10^{-4} M (Fig 1b). ALB alone, at various concentrations (10^{-4} M to 10^{-9} M), had no effect against L3 and all of the larvae had highly active movement throughout the experiment (Fig 1c).

Efficacy of combined drugs

ALB/IVM, at concentrations of $10^{-6}/10^{-6}$ M, and $10^{-5}/10^{-5}$ M, seemed to reduce larval motility more than ALB 10^{-5} M, IVM 10^{-6} M, and IVM 10^{-5} M on Day 3 and Day 2 (Fig 2a). ALB/DEC, at concentrations of $10^{-6}/10^{-6}$ M and $10^{-5}/10^{-5}$ M, seemed to reduce larval motility more than ALB 10^{-5} M, DEC 10^{-6} M, and DEC 10^{-5} M on Day 3 and Day 1 (Fig 2b). ALB/DEC seemed to have more effect than ALB/IVM at a concentration of $10^{-6}/10^{-6}$ M on Day 3 (Fig 2c).

Effect of drugs against larvae morphology after culture for 7 days

In the control group, the mean body length and width of the *B. malayi* larvae after cultivation (Day 7) increased significantly ($p < 0.05$) more than L3 before cultivation (Day 0) (2,019 and 32.2 vs 1,518 and 23.4 μ m). The larvae treated with both drugs alone and in combination, at a concentration of 10^{-6} M, had a mean body length shorter than the untreated larvae. The mean body width of the treated larvae was not significantly different compared with that of the untreated larvae.

DISCUSSION

In vitro sensitivity was tested to assess the efficacy of ALB, DEC, and IVM, using either the drugs alone or in combination, against NSP *B. malayi* (Narathiwat strain), which is endemic in southern Thailand. The concentrations of ALB, DEC, and IVM used in this study were similar to the concentrations achieved in human plasma with a therapeutic dose (Edwards *et al*, 1981; Marriner *et al*, 1986; Chiou *et al*, 1987).

It is currently known that no short chemotherapeutic treatment will clear all adult parasites (Mc Reynolds *et al*, 1993). However, many studies have assessed the efficacy of filaricidal drugs *in vivo*, eg human and animal models, to observe microfilaricidal or adulticidal activity (Nicholas *et al*, 1997; Ottensen *et al*, 1997; Bockarie *et al*, 1998; Dunyo *et al*, 2000; Shenoy *et al*, 1999, 2000, 2002). The results have shown that ALB+IVM and ALB+DEC are superior to either drug alone for reducing microfilaremia in humans. Similarly, the effect of both drug combinations in this study was superior to either drug alone, by rapidly reducing larval motility. IVM

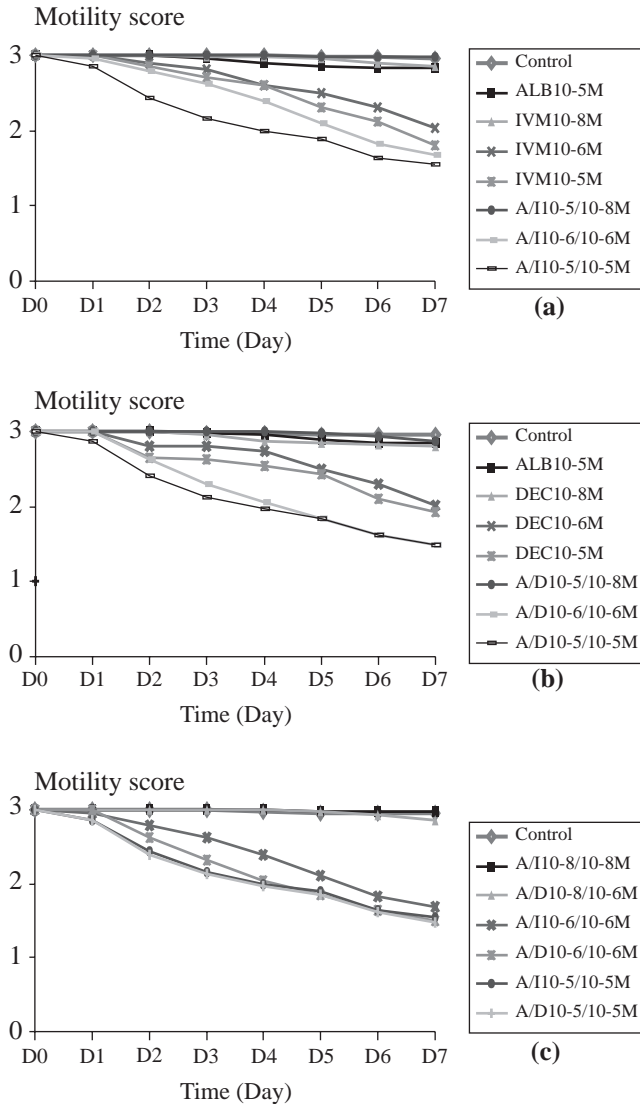


Fig 2- Motility score of NSP *Brugia malayi* L3 after being cultured with drugs combination, compared with each drug alone in NI-FBS medium for 7 days; (a) ALB/IVM (A/I); (b) ALB/DEC (A/D); (c) comparison between ALB/IVM and ALB/DEC at various concentrations (M).

and DEC alone, but not ALB, also had this effect against the larvae. Few studies reported the direct effect of filaricidal drugs against larval stage of human lymphatic filariasis in *in vitro* cultivation because of the lack of a reservoir, or laboratory-animal host, which is difficult to maintain, particularly in a small space and with concerns about animal ethics. However, the

results of an *in vivo* study showed that both DEC and IVM, when used alone or in combination, were effective against microfilariae, and ALB had some effect against the adult stage (Jayakody *et al*, 1993; WHO, 1996; Ismail *et al*, 1998).

In recent years, the *in vitro* antifilarial activity of extracts from the medicinal plant, *Cardiospermum halicacabum*, against *B. pahangi* was performed in a culture medium for 7 days (Khunkitti *et al*, 2000). The result demonstrated that a higher concentration of ethanol extract (2 mg/ml) inhibited both the motility of adult worms and the release of microfilaria by the females, and rapidly reduced the motility of microfilaria on Day 2 with an ethanol extract of 500 µg/ml. Shenoy *et al* (1999, 2000) reported that ALB+DEC seemed to have more effect than ALB+IVM with brugian microfilariae. This result was the same as this study, at a concentration of 10⁻⁶ M/ 10⁻⁶ M, on Day 3. The concentrations of DEC (3.91 ng/ml to 391 ng/ml) used in this study were less than the maximum (800-1,000 ng/ml) which is believed to be required for antifilarial activity (Stephenson and Wiselka, 2000). Since the drug had a direct effect against the parasite in the *in vitro* study, the drug concentration might be less than that of an *in vivo* study. Although the result in this study indicated that there was a synergistic pharmacodynamic adverse effect of ALB+DEC *in vitro*, no pharmacokinetic adverse effect of this drug combination has been reported *in vivo* (Shenoy *et al*, 2002). ALB sulphoxide is a pharmacologically active substance with strong antiparasite action (Dollery, 1991). ALB did not biotransform to ALB sulphoxide in the *in vitro* test. Therefore, it had no effect against the larval stage in this study. ALB alone also had no effect in this study, but it had more when combined with either DEC or IVM. This result may be the synergistic adverse effect of ALB with either IVM or DEC, because these drug combinations have greater effect than each drug alone. Morphology of the larvae was also performed to observe the effects of all drugs. The result showed that larvae treated with each drug alone or in combination at a concentration of 10⁻⁶ M, had a shorter mean body length than untreated larvae. The result indicated that IVM and DEC, at a concentration of 10⁻⁶ M, had an effect against *B. malayi* in both larval morphology and motility. However, the parasite cannot be killed, even at a high concentration (10⁻⁵ M).

Table 1
Comparative morphometric measurements (mean±SD/range) of untreated and drug-treated nocturnally-subperiodic *Brugia malayi* larvae cultured from infective third-stage larvae (L3) in NI-FBS medium for 7 days; n 20.

Day culture (drug)	Body length (µm)	Body width (µm)
0 (control)	1,518±116 (1,320-1,760)	23.4±1.6 (21.6-27.0)
7 (control)	2,019±105 ^a (1,850-2,250)	32.2±6.9 ^a (22.0-50.0)
7 (ALB, 10 ⁻⁶ M)	1,525±118 ^b (1,320-1,750)	32.6±1.8 ^a (29.7-35.1)
7 (IVM, 10 ⁻⁶ M)	1,537±160 ^b (1,210-1,760)	32.0±2.7 ^a (27.0-37.8)
7 (DEC, 10 ⁻⁶ M)	1,534±176 ^b (1,100-1,760)	32.4±3.4 ^a (27.0-37.8)
7 (ALB/DEC, 10 ⁻⁶ M/10 ⁻⁶ M)	1,539±148 ^b (1,300-1,760)	32.4±2.0 ^a (29.7-35.1)
7 (ALB/IVM, 10 ⁻⁶ M/10 ⁻⁶ M)	1,527±185 ^b (1,210-1,760)	32.4±2.8 ^a (27.0-35.1)

^aSignificant difference from Day 0 in control group, p<0.05; ^bSignificant difference from Day 7 in control group, p<0.05

Although concentrations of all drugs used *in vitro* in this study were less than *in vivo*, the results showed similar effects of the drugs in both studies. Further study of the effect of the drugs on target parasite organs, using scanning or transmission electron microscopy, to observe the surface or intra-organelle of the parasite should be conducted.

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