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ORIGINAL PAPER

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4-Methyl-7-(tetradecanoyl)-2H-1-benzopyran-2-one: a novel DNA topoisomerase II inhibitor with adulticidal and embryostatic activity against sub-periodic *Brugia malayi*

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Abstract A compound of the coumarin class, 4-methyl-7-(tetradecanoyl)-2H-1-benzopyran-2-one, was evaluated for antifilarial activity against the human filarial parasite, *Brugia malayi* (sub-periodic strain) in *Mastomys coucha*. The test compound brought about a 24.4% reduction in circulating microfilaremia on day 8 after initiation of treatment when administered by the peritoneal route at a dose of 50 mg/kg for 5 consecutive days. The compound also caused a 62.0% mortality in adult parasites. Apart from killing adult filariids, it also brought about sterilization of 81.8% of the surviving female *B. malayi*. An oral dose of 200 mg/kg for 5 consecutive days was less effective (35.5% adulticidal efficacy and 65.8% sterilization). In vitro, the compound killed adult *B. malayi* at 100 μM concentration and inhibited DNA topoisomerase II activity in the filarial parasite. Studies are in progress using the compound in combination with standard antifilarials as well as other active agents.

Introduction

Lymphatic filariasis is a major vector-borne disease of the developing world. *Wuchereria bancrofti*, the predominant lymphatic filarial parasite, infects around 120 million persons worldwide and around 44 million of these suffer from lymphoedema, elephantiasis or scrotal hydrocoel (Michael et al. 1996; Ottesen et al. 1997) while another 76 million carry parasites in their blood with hidden lymphatic and renal pathology (Ottesen 1994). Within the last 10 years, though significant progress has been made in treatment and control strategies by introducing new diagnostic and monitoring tools, single annual or biannual dose therapy, combination therapy with diethylcarbamazine (DEC), ivermectin and albendazole, lymphatic filariasis still continues to be a worsening problem due to lack of definite adulticidal efficacy in any of the above drugs. Adult parasites, which parasitize the human lymphatic system causing pathological lesions, need to be targeted to prevent progression of the disease as well as to reduce or reverse painful swelling. Benzopyrone in combination with DEC has been shown to reduce pathological swelling in filarial patients significantly (Casley-Smith et al. 1993). The findings led to synthesis of benzopyrone derivatives at our institute. An earlier publication from our laboratory revealed the adulticidal (macrofilaricidal) efficacy of a few benzopyrone derivatives against rodent filariids, *Acanthocheilonema viteae* and *Litomosoides carinii* (Tripathi et al. 2000). These compounds also inhibited filarial topoisomerase II (Tripathi et al. 2001). While screening a number of benzopyrone (coumarin) derivatives, one compound, 4-methyl-7-(tetradecanoyl) 2H-1-benzopyran-2-one, has been found to inhibit DNA topoisomerase II in filarial parasites. DNA topoisomerase II has earlier been reported to be an important antifilarial drug target (Tripathi et al. 2000). This compound was therefore tested against the human lymphatic filarial parasite *Brugia malayi* in vitro and in vivo in a rodent host, *Mastomys coucha*, to assess its antifilarial activity against circulating microfilariae and adult parasites. The present investigation also reports the effect of compound on filarial DNA topoisomerase II.

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Materials and methods

Host parasite model

M. coucha infected with sub-periodic strain of *B. malayi* was used for screening the above-mentioned compound.

Infection

The sub-periodic strain of *B. malayi* was maintained in the rodent *M. coucha* through cyclical transmission by the vector *Aedes aegypti* (Singh et al. 1997). Briefly, mosquitoes fed on 1% glucose solution were infected by feeding on the blood of a heavily microfilaremic donor *Mastomys*. Infective larvae were isolated from fed mosquitoes and were washed and used for infecting 8-week-old male *Mastomys* (out-bred) reared and bred in the animal house of the Central Drug Research Institute (CDRI).

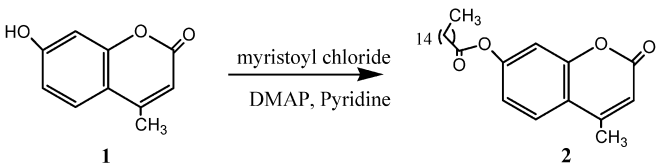
Each *Mastomys* received 100 infective larvae by the subcutaneous route into the neck region. Gerbils (*Meriones unguiculatus*) were infected by intraperitoneal inoculation of 200 infective larvae (L_3) of *B. malayi*.

Synthesis of 4-methyl-7-(tetradecanoyl)-2H-1-benzopyran-2-one

A solution of 7-hydroxy 4-methyl coumarin (7.0 g, 39.77 mmol) in anhydrous pyridine (25.0 ml) was magnetically stirred at ice bath temperature. To it was added *N,N*-dimethylamino pyridine (DMAP) (200 mg) followed by addition of myristoyl chloride (10 ml, 36.78 mmol) slowly. The reaction mixture was stirred at the same temperature for 30 min then at room temperature for 18 h. It was poured over crushed ice (100 g) and extracted with chloroform (2×100 ml). The organic layer was dried (over Na_2SO_4) and evaporated under reduced pressure to give a crude mass, which was purified over a SiO_2 column using CH_2Cl_2/MeOH (98:02) as eluant to give the desired compound (3.5 g) (Scheme 1). PMR δ values in $CDCl_3$ were as follows: 7.70 (d, J = 8.8 Hz, 1H, H-5); 7.09 (dd, J = 8.4 Hz, 2.2 Hz, 2H, H-6 and H-8; 6.36 (s, 3H, H-3); 2.59 (t, J = 7.5 Hz, 2H, $-CH_2$ CO–); 2.31 (s, 3H, CH_3); 1.76 (m, 4 H, 2×CH_2); 1.26 (m, 18 H, CH_2); 0.88 (t, J = 6.6 Hz, 3H, CH_3). For ^{13}C NMR in $CDCl_3$, δ values were as follows: 171.9, 160.4, 154.58, 158.63, 152.29, 125.71, 118.48, 118.11, 114.82, 110.80, 34.72, 32.30, 30.02, 29.97, 29.73, 29.61, 29.45, 25.18, 23.06, 19.04, 14.07. The determined melting point was 72–74°C.

Treatment schedule of test compound and reference drug

DEC was included as a reference drug during the study. The compound and reference drug were pulverized to fine power and fine suspensions were made in distilled water containing 0.1% Tween-80. The test compound was administered intraperitoneally at 50 mg/kg body weight as well as orally at 200 mg/kg body weight for 5 consecutive days, while DEC was fed orally at 25 mg/kg. The drug suspensions were freshly prepared daily within an hour of drug administration.



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Scheme 1 Synthesis of 4-methyl-7-(tetradecanoyl)-2H-1-benzopyran-2-one

Assessment of in vitro efficacy

The efficacy of compound was assessed on adult female *B. malayi*. In vitro testing was done as described earlier (Mukherjee et al. 1997). Keeping untreated worms as control, test compound and DEC were used at two concentrations, i.e. 100 and 50 µM. Adult *B. malayi* were isolated from the peritoneal cavity of gerbils between 120 and 180 days after L_3 inoculation. The adult female worm was incubated at 37°C in the presence of the compound for 24 h in medium RPMI-1640 containing 1% glucose and antibiotic-antimycotic mixture (Sigma, USA). Worms were also exposed to DEC, the reference drug in the same way. The effect of compound was assessed by microscopic observation of worm motility and assessing the worm viability by percentage inhibition of the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) (Mukherjee et al. 1998) following 24-h drug exposure. Microscopic observation of worm motility was made both for treated and control parasites. Motility was expressed as follows: high (5 + /4 +); moderate (3 +); low (2 +); sluggish (1 +) and dead (D). At the end of incubation, parasites were individually transferred to phosphate-buffered saline (PBS) to observe the change in motility (if any), blotted quickly on filter paper and transferred to 100 µl PBS containing 0.25 mg MTT/ml for 30 min at 37°C. Each worm was later transferred to 200 µl DMSO in a 96-well plate; this was allowed to stand at room temperature for 1 h and absorbance was determined at 510 nm. More than 50% inhibition of MTT reduction by treated parasite compared to untreated control was considered as adulticidal action.

Assessment of in vivo efficacy

Micro- and macrofilaricidal efficacy were evaluated as described earlier (Misra et al.1984). Blood smears were made just before initiation of treatment (day 0), on days 8 and 15 and thereafter at fortnightly intervals till day 90. Any decrease or increase in microfilarial count in comparison to pretreatment level was expressed as a percentage change in microfilaremia. The treated and control *Mastomys* were autopsied under deep anesthesia and various tissues harboring adult parasites (lungs, heart, testes) were recovered and teased gently in PBS to recover adult parasites. The number and condition of worms was assessed. At least six surviving female worms were kept individually on a glass slides in a drop of PBS and teased with needles to allow the uterine contents to come out of the uteri. The adverse effect on intrauterine stages, if any, of compound or DEC was examined microscopically. Female worms with no uterine contents or uteri carrying dead or degenerated developing stages including free released microfilariae or even females with only early eggs but no further development were considered sterilized.

Estimation of DNA topoisomerase II

Adult parasites of *Setaria cervi* were obtained from a local abattoir and brought in the laboratory in 0.85% NaCl. Adult *B. malayi* was recovered from the peritoneal cavity of gerbils infected intraperitoneally 90–180 days previously. Worms were thoroughly washed in saline and homogenized in homogenizing medium (10% homogenate). Protein content was measured by the method of Lowry et al. (1951).

The reaction catalyzed by DNA topoisomerase II was estimated as reported earlier (Pandya et al. 1999). The reaction mixture in a final volume of 20 µl contained the following: 50 mM Tris.HCl, pH 7.5; 50 mM KCl; 10 mM $MgCl_2$; 1 mM ATP; 0.1 mM EDTA; 0.5 mM dithiothreitol (DTT); 30 µg/ml bovine serum albumin (BSA); 0.25 µg pBR322 DNA; and enzyme protein. The reaction was carried out at 37°C for 30 min. and stopped by adding 5 µl stop buffer. The samples were subjected to electrophoresis on 1% agarose gel in Tris-acetate buffer for 18 h at 20 V. Gels were stained with ethidium bromide (0.5 µg/ml) and visualized and photographed on a GDS 7500 UVP Trans illuminator (Ultraviolet Products, UK). Incubating enzyme protein with the inhibitor for

10 min. at 37 °C and starting the reaction by addition of pBR322 measured the effect of inhibitors on the enzyme activity. The percentage inhibition was measured by micro densitometry of the gel with the gel base/gel blot Progel analysis software program.

Statistical analysis

The Newman-Keuls multiple comparison test was used to analyze the statistical significance of the data.

Results

Efficacies

The in vitro and in vivo antifilarial efficacies of the compound and the reference drug DEC are presented in Tables 1 and 2, and in Figs. 1, 2 and 3.

Table 1 In vitro adulticidal efficacy of test compound and diethylcarbamazine (DEC) against adult female *Brugia malayi*

Agent	Concentration	Motility[ab]	Percentage inhibition of MTT[c] reduction[b]	Active/ inactive[b]
Compound	100 μM	D	59.5	Active
Compound	50 μM	3+	3.6	Inactive
DEC	100 μM	3+	Nil	Inactive
DEC	50 μM	4+	Nil	Inactive

[a]Motility of worm was graded as 4+ (highly motile), 3+ (motile), 2+ (less motile), 1+ (sluggish) or (D) non-motile
[b]Compound was considered to be active if it rendered worms non-motile or caused 50% or more inhibition in reduction of MTT compared to untreated worms
[c]*MTT* 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide

In vitro efficacy

The test compound killed adult *B. malayi* in vitro at 100 μM concentration by making the worm completely non-motile, and there was 59.5% inhibition in MTT reduction by treated adult females at this concentration. A lower concentration of 50 μM was not effective (Table 1).

In vivo efficacy

Effect on microfilaremia

The test compound caused 24.4% reduction in circulating microfilaremia on day 8 after commencement of treatment when administered by peritoneal route to *Mastomys* (Table 2). The microfilarial counts rose thereafter. However, a 200 mg/kg dose by the oral route proved ineffective against circulating microfilariae. On the other hand, the reference drug, DEC, proved to be a highly effective microfilaricide, bringing about an 81.3% fall in the microfilarial count on day 8 compared to the pretreatment microfilarial level. Recurrence of microfilaremia in the latter started from day 45 onwards.

Adulticidal (macrofilaricidal) efficacy

The test compound at 50 mg/kg by the intraperitoneal route brought about 62.0% mortality of adult *B. malayi* compared with the untreated control. Of the two sexes, female worms were more affected (61.1%) than their male counterparts (56.8%) (Fig. 3). Apart from the death of adult worms, an average of 81.8% of the surviving females showed sterilization when their uterine contents were microscopically observed (Fig. 2). An oral dose of 200 mg/kg appeared less effective; it brought

Table 2 Effect of the test compound and reference drug on microfilaremia

Agent	Dose (schedule)	Percent change (mean ± SD) in microfilaraemia/10 μl blood at different days over pretreatment level						
		Day 8	Day 15	Day 30	Day 45	Day 60	Day 75	Day 90
Compound	50 mg/kg intraperitoneally for 5 days	−24.4 ± 12.3	+ 2.8 ± 25.2	+ 88.5 ± 50.9	+ 84.4 ± 43.7	+ 105.3 ± 69.5	+ 104.6 ± 64.4	+ 56.3 ± 19.0
Compound	200 mg/kg by mouth for 5 days	+ 50.3 ± 102.8	+ 62.9 ± 112.5	+ 165.0 ± 207.7	+ 414.3 ± 684.7	+ 391.4 ± 719.3	+ 635.2 ± 962.4	+ 570.7 ± 837.6
DEC	50 mg/kg by mouth for 5 days	−81.3 ± 11.2	−78.5 ± 10.3	−56.8 ± 31.6	+ 10.8 ± 23.5	+ 21.1 ± 62.5	+ 3.2 ± 58.4	+ 38.4 ± 77.4
Control (for intraperitoneal dose)	Nil	+ 58.1 ± 67.0	+ 183.3 ± 199.4	+ 298.7 ± 203.2	+ 402.5 ± 223.9	+ 556.9 ± 362.6	+ 664.8 ± 398.0	+ 759.3 ± 512.9
Control (for oral dose)	Nil	+ 89.4 ± 114.0	+ 196.3 ± 273.2	+ 230.0 ± 324.4	+ 322.1 ± 361.2	+ 531.8 ± 573.2	+ 835.8 ± 1046.7	+ 782.6 ± 802.2

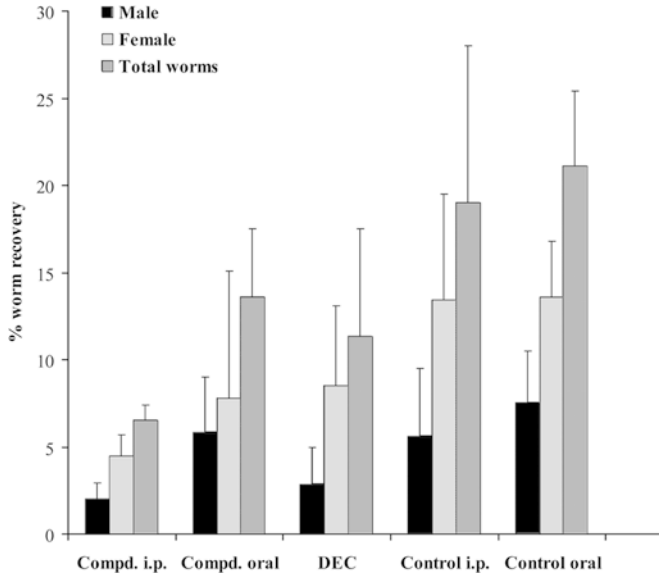

Fig. 1 Adult *Brugia malayi* recovery (mean ± SD) from *Mastomys* after treatment with test compound and the reference drug

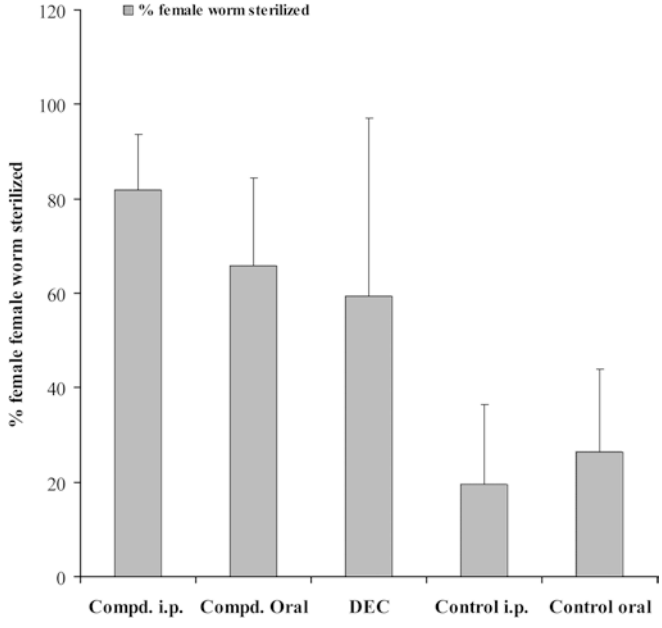

Fig. 2 Effect of compound and reference drug on sterilization of female *B. malayi*

about 35.5% mortality of adult worms (Fig. 3) with 65.8% female sterilization (Fig. 2). Here also, female worms were more affected (43%) than the males (23.3%) when compared with the untreated control worms. DEC, the standard drug, appeared less effective (46.7% macrofilaricidal efficacy) than the given compound and caused only 59.4% female sterilization. Female and total worm recovery for the test compound administered intraperitoneally was less than that for DEC, the standard filaricide (Fig. 1), however, the difference was not statistically significant ($P > 0.05$). The statistical analysis

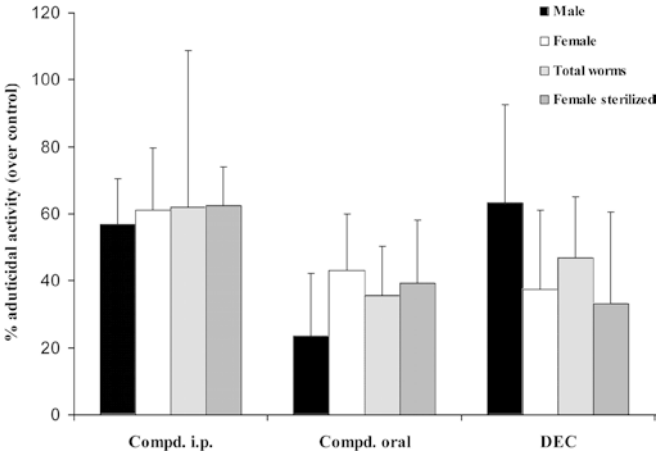

Fig. 3 Macrofilarial activity (mean ± SD) of the test compound and the reference drug against *B. malayi* in *M. coucha*

Table 3 Statistical analysis of macrofilaricidal activity of test compound and reference drug by the Newman-Keuls multiple comparison test

Group	Percentage worm recovery			Percentage of surviving female worms sterilized
	Male	Female	Total	
A versus B	ns	ns	*	ns
A versus C	ns	ns	ns	ns
A versus D	ns	**	**	**
A versus E	**	**	**	**
B versus C	ns	ns	ns	ns
B versus D	ns	ns	ns	**
B versus E	ns	ns	*	**
C versus D	ns	ns	*	**
C versus E	**	ns	**	**
D versus E	ns	ns	ns	ns

*Significant ($P < 0.05$), **highly significant ($P < 0.01$), *ns* non-significant ($P > 0.05$)

of the adulticidal efficacy of test compound is presented in Table 3.

Inhibition of topoisomerase II activity in vitro

The test compound inhibited the topoisomerase II activity of the filarial parasite *S. cervi* in vitro by 75% and 90% at 10 and 20 μg concentrations, respectively. The infected host was also treated with the test compound (200 mg/kg by mouth for 5 days) and worms recovered 90 days after initiation of treatment were tested for DNA topoisomerase II activity in vitro. The treated worms showed little action by DNA topoisomerase II as compared to untreated control parasites.

Discussion

Both available filaricides, DEC and ivermectin, are able to eliminate microfilaremia in human lymphatic filariasis

(Ottesen et al. 1997), but the adult parasites mostly remain unaffected with the result there is relapse of microfilaremia and no relief from the filarial disease lesions. An antifilarial drug is required which can kill the adult parasites and may also reverse the pathology. Coumarins or benzopyrones have recently been identified as affecting lymphodema (Casley-Smith and Casley-Smith 1992; Casley-Smith et al. 1993), apart from their effect on adult parasites (Tripathi et al. 2000). In a bid to develop such derivatives, some compounds from our laboratory were shown earlier to have activity on adult filarial parasites of rodent species (Tripathi et al. 2000). In the present investigation we evaluated one compound of the coumarin class against the human filarial parasite, *B. malayi*, for its antifilarial activity, and this compound revealed strong macrofilaricidal efficacy with mild effect on circulating microfilaremia. The compound also caused sterilization of some of the surviving female worms. In vitro testing also revealed its adulticidal property at 100 μM concentration. The test compound also strongly inhibited DNA topoisomerase II enzyme activity when tested on the adult bovine filariid *S. cervi*. The bovine filaria was used for in vitro test because of the easy availability of this species. Although the test compound strongly inhibited filarial DNA topoisomerase II in vitro, we could not find significant inhibition when the worms were taken from *Mastomys* treated with the test compound. It is important to mention here that these worms were exposed to the test compound long ago, i.e. the animals were autopsied on day 90 after initiation of 5-days treatment, and by that time the effect on enzyme activity in these worms would have disappeared. In the case of target human filarial parasites inhabiting the lymphatic system, there is difficulty in delivering the compound to the target site. The present benzopyrone derivative was synthesized by attaching the lipophilic chain to the compound with an aim of targeting the compound at the location preferred by the adult worm. In *Mastomys*, though the majority of the inoculated infective larvae of *B. malayi* establish themselves as adult parasites inside various tissues such as lungs, heart and testes, the remaining ones parasitize the lymphatic system. It appears quite likely that the target human filarial parasites inhabiting only the lymphatic system would be more susceptible to this compound. The compound showed significantly superior efficacy by the intraperitoneal route in terms of micro- and macrofilaricidal action as well as female worm sterilization ($P < 0.01$) over untreated control groups. The oral route of administration was also significantly effective in killing adult *B. malayi* or sterilizing the female parasites when comparison was made with its respective control group. The oral administration did not affect circulating microfilaremia even though the dose was much higher. This shows that there might be some problem with the absorption of the compound through the gastrointestinal tract, resulting in lower antifilarial efficacy of the compound by the oral route. Although the action of compound (by the intraperitoneal route) on peripheral microfilaremia was low (on day 8) and short lived, microfilarial concentration was significantly low when compared with the untreated controls. The most interesting part was a further fall in microfilarial level at the end of observation (day 90) though it still crossed the day-0 level. It is quite possible, had the observations continued for longer period, that microfilaremia would have further fallen. Thus the suppressed microfilaremia at the end of observation period may be attributed to a combined effect of adult worm killing and embryostatic action of the compound. This macrofilaricidal property, associated with some microfilaricidal and a remarkable embryostatic action combined with the probable anti-inflammatory property of known benzopyrone compounds (Casley-Smith and Casley-Smith 1992; Casley-Smith et al. 1993), makes this compound a very promising one. A combination trial using this compound along with the standard filaricides diethylcarbamazine or ivermectin appears worth attempting and is currently under way in our laboratory using the same host-parasite model. Thus compound 4-methyl-7-(tetradecanoyl)-2H-1-benzopyran-2-one developed by CDRI provides a novel lead for synthesis and development of new antifilarial agents, which may kill micro- and macrofilariae, cause worm sterilization and possibly may also alleviate human suffering caused by this disease.

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