

Chemotherapeutic Studies on *Litomosoides carinii* Infection of *Mastomys natalensis**

1. The Filaricidal Action of 2,6-bis-Benzimidazoles

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The antifilarial action of 2-[2-(4-hydroxyphenyl)-6-benzimidazolyl]-6-(1-methyl-4-piperazyl)benzimidazole (HOE 33258) was investigated in Mastomys natalensis infected with Litomosoides carinii. The subcutaneous administration of HOE 33258 in a single daily dose for 5 consecutive days, or at other intervals, produced, depending on the dosage, a rapid reduction in the number of microfilariae in the circulating blood. The reduction amounted to more than 90% within 7-14 days after the treatment was started or at the end of the dosage schedule. The small, slow increase in the microfilarial count during a period of 6-7 weeks after treatment ended reached not more than half the number present before treatment. HOE 33258 showed marked activity on the reproductive system of mature female worms, although only few macrofilariae were killed by the drug. The results also demonstrated the usefulness of L. carinii infection of M. natalensis as a model for the evaluation of the filaricidal activity of drugs.

In the search for new filaricidal compounds, systematic screening tests have been carried out during the past 25 years, almost entirely on *Litomosoides carinii* in cotton rats (*Sigmodon hispidus*). Several investigations have provided useful information in relation to the biology of untreated infections as well as to the effects of different types of chemical compounds in naturally acquired or experimental filariasis in cotton rats.

In extensive studies on the susceptibility of *Mastomys natalensis* to parasitic helminths (Texdorf, 1967; Zahner, 1967; Lämmler et al., 1968b, 1969, 1970), this animal proved to be an excellent final host also for the cotton rat filaria, *L. carinii* (Lämmler et al., 1968a, 1969; Pringle & King, 1968; Zahner et al., 1970a, 1970b). It was therefore of great interest to evaluate the activities of various well-known or newly developed filaricidal drugs against microfilariae or adult parasites in this new laboratory animal. In a recent publication, Foster et al. (1969) reported, for

the first time, the usefulness of the *L. carinii* infection of *M. natalensis* for chemotherapeutic experiments.

This paper describes an investigation on the antifilarial activity of 2-[2-(4-hydroxyphenyl)-6-benzimidazolyl]-6-(1-methyl-4-piperazyl)benzimidazole (HOE 33258),³ which was discovered and described by Loewe et al. (1967), Lämmler & Raether (1968) and Loewe & Urbanietz (1968), working with cotton rats infected with *L. carinii*.

The compound, which appears to be also a useful fluorochrome for staining animal-cell nuclei (Herzog & Schütze, 1968; Lämmler & Schütze, 1969), has been found to be one of the most effective filaricidal substances of a series of basically substituted bis-benzimidazoles (Loewe et al., 1967; Loewe & Urbanietz, 1968; Raether & Lämmler, 1971).

MATERIALS AND METHODS

For propagating the *L. carinii* infection in *M. natalensis*, homogeneous colonies of the rat mite *Ornithonyssus bacoti* were infected with the larval stages of *L. carinii* by allowing them to feed on several

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³ Preparation HOE 33258 was supplied by Farbwerke Hoechst AG, Frankfurt am Main, Federal Republic of Germany.

cotton rats or *M. natalensis* which had numerous microfilariae in their peripheral blood. The animals used in this study were bred by conventional methods in a small breeding station at the Institute; they were fed on a diet developed for Syrian hamsters and kept in small groups in Macrolon cages.¹ Drinking water was available *ad libitum*. At the end of the pre-patent period, the infected animals were examined quantitatively for microfilariae in the peripheral blood twice before treatment and thereafter at weekly or 2-weekly intervals for 70 or 100 days, using the method of Raether & Meyerhöfer (1967).

¹ Polycarbonate cages, supplied by PAG Presswerk AG, Essen, Federal Republic of Germany.

Groups of 6–10 animals were treated with HOE 33258 in form of the diphosphate suspended in water, the trihydrochloride dissolved in water, or the free base suspended in oil, in single subcutaneous doses daily for 5 consecutive days, starting about 90 days after infection. The animals were necropsied 70 or 100 days after the beginning of treatment. The adult filariae were removed from the pleural and peritoneal cavities, counted, and examined for mobility and viability. The reduction in numbers of microfilariae in treated *M. natalensis* was evaluated in comparisons with infected control animals, the average number of microfilariae per group before treatment being taken as 100.

Table 1. Chemotherapeutic activity of subcutaneous administration of HOE 33258 as the diphosphate, the trihydrochloride, and as the free base against *Litomosoides carinii* in *Mastomys natalensis*

Dosage (mg/kg × 5)	No. of animals		Percentage reduction of microfilarial count the following number of days after the start of treatment: ^a							Average no. of microfilariae at necropsy			Encapsulation ^b
	Start	End	3	7	14	21	42	70	100	Females	Males	Total	
Diphosphate salt suspended in water													
2.5	6	6	92.8	89.0	61.0	62.2	60.3	47.5		13	11	24	0
5	6	6	98.1	97.1	82.5	92.3	93.3	88.1		12	8	20	0
10	6	6	87.1	99.5	98.1	96.7	93.1	62.6		15	9	24	0
20	6	6	99.8	100	99.9	99.5	99.3	95.1		26	43	69	0
40	10	8	72.4	98.2	99.8	99.8	98.7	93.9	79.9	24	17	41	5
60	11	9	73.9	98.5	99.8	99.6	92.9	79.5	53.4	25	16	41	7
Control	7	7	0	0	0	0	14.2	6.6		31	29	60	0
Control	4	3	9.6	23.4	15.2	5.2	1.2	27.6		12	8	20	0
Trihydrochloride salt dissolved in water													
10	6	6		64.0	80.0	88.0	84.0	67.0	72.0	2	2	4	0
20	4	3		96.2	99.3	97.6	84.5	85.9	72.6	25	37	62	0
40	10	9	80.4	93.0	100	100	67.0	42.6	17.3	17	14	31	6
60	10	8	84.7	97.3	100	98.7	99.0	93.7	82.0	6	5	11	8
Control	6	6	0	0	0	0	0	0	0	13	9	22	0
Free base suspended in oil													
2.5	10	9	0	0	9.3	0	2.6	0	0	10	10	20	0
5	10	10	0	43.1	0	0	0	0	0	16	13	29	0
10	10	10	13.1	89.2	91.3	50.3	42.9	32.6	30.3	7	6	13	0
20	10	8	0.1	0	87.0	68.3	67.5	24.6	29.0	16	10	26	0
40	10	10	47.1	99.0	96.9	97.1	93.2	90.5	84.0	16	34	50	8
Control	6	6	0	0	0	0	0	0	0	13	9	22	0

^a The percentage reduction of microfilariae was calculated on the basis of the average number of microfilariae before the treatment.

^b Number of animals with microfilariae encapsulated in fibrin.

RESULTS

Efficacy of HOE 33258 against microfilariae

The subcutaneous administration of HOE 33258, using the aqueous suspension of the diphosphate in single daily doses as low as 2.5 mg per kg of body weight during 5 consecutive days, produced a marked reduction in the number of microfilariae in the peripheral blood. A maximum decrease was noticed between the third and the seventh days from the beginning of treatment (Table 1, Fig. 1). This reaction was followed by an increase of the microfilarial count to half the number present before treatment. The higher dosages between 5×10 mg/kg and 5×60 mg/kg given subcutaneously produced falls in the microfilarial count of more than 90% for 42 days or longer, but even these dosages did not remove the microfilariae from the circulating blood completely and the same dosages did not prevent a subsequent increase in number. The maximum reduction in numbers of microfilariae was observed about 14 days from the beginning of treatment with 5×40 mg/kg and 5×60 mg/kg, given subcutaneously.

The treatment of the *L. carinii* infection in *M. natalensis* with equal doses of the compound in the form of the soluble trihydrochloride produced a similar effect, whereas the injection of the same dosages in

the form of the free base suspended in oil showed an irregular and inadequate activity. The microfilaricidal effect of the water-soluble or water-suspended salts of the compound was more or less proportional to the dose applied.

The subcutaneous administration for 5 days of the substance in the form of the diphosphate in the same daily dose of 10 mg/kg, but at different intervals (Fig. 2), caused also a considerable fall in the microfilarial count. A maximum reduction of more than 95% in the number of microfilariae in the circulating blood could be observed, depending on the schedule, a few days after the treatment had ceased. Even these regimens did not prevent a slight increase again in the numbers of microfilariae after various intervals.

Efficacy of HOE 33258 against macrofilariae

The administration of HOE 33258 in the form of the two salts or as the free base suspended in oil did not reveal any marked macrofilaricidal activity. Only when high dosages were applied subcutaneously (5×40 mg/kg and 5×60 mg/kg) was a small number of adult parasites killed and encapsulated in fibrin (Table 1). No difference in the activity of HOE 33258 could be seen between males and females of *L. carinii*.

Investigations on the viability of macrofilariae and on the production of microfilariae by the females

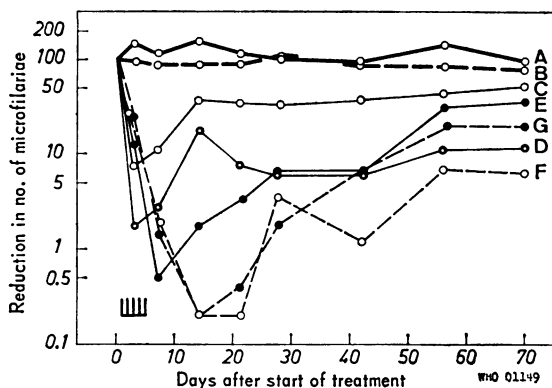


Fig. 1. Microfilaricidal effectiveness of HOE 33258 diphosphate in *M. natalensis* infected with *L. carinii* after the administration of 5 consecutive daily subcutaneous doses. The reduction in number of microfilariae is based on an average number of 100 before treatment. The dosages were as follows: C: 5×2.5 mg/kg; D: 5×5 mg/kg; E: 5×10 mg/kg; F: 5×40 mg/kg; G: 5×60 mg/kg. Curves A and B represent the control groups for groups C–D–E and F–G, respectively.

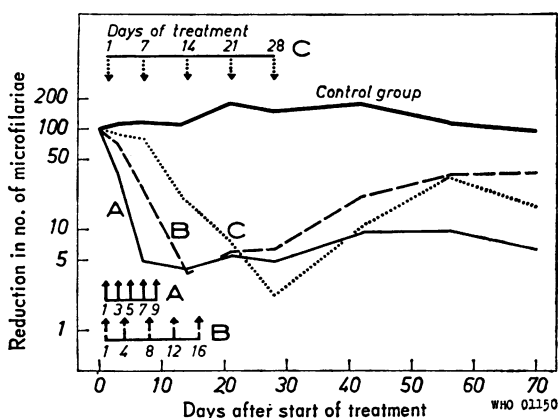


Fig. 2. Microfilaricidal effectiveness of HOE 33258 diphosphate in *M. natalensis* infected with *L. carinii* after the administration of 5 daily subcutaneous doses at different intervals. The reduction in number of microfilariae is based on an average number of 100 before treatment. The dosage was 5×10 mg/kg.

Table 2. Viability of macrofilariae and production of microfilariae after treatment ^a with a preparation of HOE 33258 ^b

		Results at necropsy the following no. of days after the start of treatment:						
		7	14	21	28	35	49	63
Treated groups ^e	Macrofilariae ^c	+++	+++	+++	++	+++	+++	+
	Microfilariae ^c	++	+	++	+++	+++	+++	++
	Embryos ^c	+++	+	++	+++	+++	+++	++
	Average no. of living microfilariae in culture	764	826	580	2 020	1 906	2 100	3 220
	Percentage reduction of microfilariae in blood:							
	individual groups	95.6	96.4	100	99.8	87.1	94.0	75.0
	no. of animals	21	18	15	12	9	6	3
	total	99.5	99.1	99.9	99.1	90.1	83.0	59.6
Control groups ^e	Macrofilariae ^c	+++	+++	+++	+++	+++	+++	+++
	Microfilariae ^c	+++	+++	+++	+++	+++	+++	+++
	Embryos ^c	+++	+++	+++	+++	+++	+++	+++
	Average no. of living microfilariae in culture	8 086	8 053	2 080	13 755	7 750	9 560	6 210
	Percentage reduction of microfilariae in blood:							
	individual groups	0	0	0	0	0	0	0
	no. of animals	21	18	15	12	9	6	3
	total	0	0	0	0	0	0	0
	Percentage reduction in numbers of microfilariae produced by female worms of treated animals <i>in vitro</i>	90.6	89.8	72.2	85.4	75.5	78.1	48.2

^a Dosage: 5 × 20 mg/kg subcutaneously.

^b This formulation of the trihydrochloride contained 0.5 % of lidocaine.

^c + = Large proportion of immobile or dead macrofilariae, microfilariae or embryos; ++ = some immobile or dead macrofilariae, microfilariae or embryos; +++ = normal populations of living macrofilariae, microfilariae and embryos.

^d A few macrofilariae were found to be encapsulated in fibrin.

^e A total of 3 animals per group and per week.

after treatment with HOE 33258 in subcutaneous doses of 5 × 20 mg/kg demonstrated that the reproduction rate of female worms was markedly decreased (Table 2). The production of microfilariae by 2 female worms isolated from the treated animals and incubated in 0.8 ml of phosphate-buffered saline (pH 6.5) and 0.2 ml of *Mastomys* serum at 37°C for 24 hours was considerably reduced for several weeks compared with the number of microfilariae produced by 2 females from untreated animals under the same conditions. This long-term effect appears to be related to the large reduction in numbers of circulating microfilariae in the blood of the host. Microscopical examination of the reproductive sys-

tem of female worms separated from treated animals showed numerous deformed and degenerated embryos 2, 3, and 9 weeks after the beginning of the treatment.

DISCUSSION

The subcutaneous administration of HOE 33258 in the form of the diphosphate suspended in water in a single daily dose of 2.5 mg/kg for 5 consecutive days produced a rapid decrease of more than 90% in the microfilarial count in the circulating blood during the first 7 days after the beginning of treatment. Higher doses of the compound administered in the same schedule intensified the reduction in number of microfilariae gradually by more than 99%

between 14 and 21 days. After this period, a reduction in the number of microfilariae of more than 90% was observed to last for 6 weeks or longer (Fig. 1, Table 1). The results demonstrate an important difference in the effectiveness of the basically substituted 2,6-bis-benzimidazoles in comparison with that of diethylcarbamazine (Lämmler et al., 1971). The latter compound, in an oral dosage 10 times as high as the subcutaneous dose of HOE 33258 administered in the current tests, caused a similar, but smaller, reduction in the number of microfilariae in the circulating blood and this fall was followed by a rapid rise within 2 weeks.

When HOE 33258 was administered subcutaneously in 5 daily doses of 10 mg/kg at days 1, 3, 5, 7, 9, days 1, 4, 8, 12, and 16 or days 1, 7, 14, 21, and 28, the fall in the microfilarial count was slower, depending on the schedule, and the maximum reduction of more than 95% could always be observed directly after the treatment ended (Fig. 2).

The fact that the subsequent increase in number of microfilariae in the circulating blood after treatment never reached half the number before the treatment might be due either to the amount of HOE 33258 in the blood and tissues or to the action of the drug on the reproductive system of female worms.

The strong depression of microfilarial production by female worms isolated from treated animals in weekly or 2-weekly intervals and kept in *in vitro* culture for 1 day (Table 2) demonstrated the influence of the drug also upon the mature parasites. The similarity between the high percentage reduction of microfilariae for 7-9 weeks in the circulating blood and the lowering of the microfilarial production by treated female worms in *in vitro* culture after the administration of HOE 33258 trihydrochloride in daily subcutaneous doses of 20 mg/kg for 5 consecutive days (Table 2) proved that HOE 33258 affected mature worms, although only a few dead macrofilariae encapsulated in fibrin could be found.

The irregular and inadequate activity of the compound in the form of the free base suspended in oil might depend on slow or irregular absorption or on local irritation of the tissues.

The results of these studies of *L. carinii* infection in *M. natalensis* have demonstrated that, in comparison with infections in cotton rats, there are some differences (Raether & Lämmler, 1971). The subcutaneous administration of HOE 33258 diphosphate in the same dosages to cotton rats infected with *L. carinii* not only produced a higher per-

centage reduction of microfilariae in the circulating blood but also showed a stronger activity against adult parasites. Such an effect could be observed after the subcutaneous administration of doses as low as 5×4 mg/kg and 5×8 mg/kg. The higher susceptibility of macrofilariae in cotton rats, especially of the female worms, was linked with a strong action of the drug on the reproductive system, as well as with the deaths of worms and their subsequent encapsulation in fibrin (Raether & Lämmler, 1971).

HOE 33258 was found to persist for a long time in the tissues of cotton rats and *M. natalensis* treated with the drug. Fluorescence microscopical studies of blood-smears and tissue sections from treated animals revealed bright fluorescence of the cell nuclei of microfilariae as well as of the blood leucocytes and cell nuclei in host tissue and organs. In smears and histological sections of liver and kidney, relatively strong fluorescence of the nuclei could be recognized up to 37 days after treatment, or longer (Lämmler & Schütze, 1969). However, it cannot be concluded from this observation that the filaricidal effect over a period of several weeks depends entirely on the presence of HOE 33258 in cell nuclei of host tissues and organs. It seems that both the action of the drug on female worms and the residual HOE 33258 in the tissues and organs together influenced the numbers of microfilariae in the circulating blood.

The present studies on the filaricidal activity of HOE 33258 in *M. natalensis* infected with *L. carinii* confirmed the excellent results obtained in chemotherapeutic experiments made previously on cotton rat filariasis (Lämmler & Raether, 1968; Raether & Lämmler, 1971). The compound proved to be highly effective against microfilariae and showed a marked influence on the reproduction rate of mature female worms. Furthermore, the results demonstrated that *L. carinii* infection in *M. natalensis* is a useful and valuable model for evaluating the chemotherapeutic activity of filaricidal drugs in laboratory experiments.

The effectiveness of HOE 33258 against other filaria species was also recognized. The subcutaneous administration of the compound in the form of the trihydrochloride dissolved in water was highly effective against *Dirofilaria immitis* in a dog, against *Tetrapetalonema marmosetae* (Faust, 1935) in two tamarins (*Saguinus oedipus*), and against an unidentified species of microfilaria in a gorilla (Lämmler, Schütze & Herzog, unpublished data), without any side-effects.

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RÉSUMÉ

ÉTUDES SUR LA CHIMIOTHÉRAPIE DE L'INFECTION PAR *LITOMOSOIDES CARINII*
CHEZ *MASTOMYS NATALENSIS* : 1. ACTION FILARICIDE D'UN BIS-BENZIMIDAZOLE-2,6

On a cherché à déterminer l'activité antifilarienne du diphosphate de [(hydroxy-4 phényl)-2 benzimidazolyl-6]-2 (méthyl-1 pipérazyl-4)-6 benzimidazole (HOE 33258) chez *Mastomys natalensis* infecté par *Litomosoides carinii*.

Administré quotidiennement par voie sous-cutanée à la dose de 2,5 mg par kilo de poids corporel pendant 5 jours consécutifs, ou à des doses supérieures et à intervalles plus longs, le composé entraîne une diminution rapide du nombre des microfilaries dans le sang circulant. La réduction de la densité microfilarienne dépasse 90%

dans les 7 à 14 jours suivant le début du traitement. Les numérations pratiquées 6 à 7 semaines après le traitement ne montrent qu'une réapparition lente et modérée des microfilaries dont le nombre reste inférieur de moitié au nombre initial. Bien que ne provoquant qu'une faible mortalité parmi les macrofilaires, le HOE 33258 agit de façon nette sur la fonction de reproduction chez le ver femelle adulte.

Cette étude [montre que l'infection de *M. natalensis* par *L. carinii* peut servir de modèle expérimental pour l'évaluation de l'activité filaricide de composés chimiques.