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[^{3}H]PARAHERQUAMIDE BINDING TO *CAENORHABDITIS ELEGANS*

STUDIES ON A POTENT NEW ANTHELMINTIC AGENT

JAMES M. SCHAEFFER,*† TIMOTHY A. BLIZZARD,‡ JOHN ONDEYKA,‡ ROBERT GOEGELMAN,‡ PETER J. SINCLAIR‡ and HELMUT MROZIK‡

Departments of *Biochemical Parasitology and ‡Chemistry, Merck Sharp & Dohme Research Laboratories, Rahway, NJ 07065, U.S.A.

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Abstract—Paraherquamide was identified recently as a potent anthelmintic agent. In this paper we describe the identification and characterization of a specific, high-affinity paraherquamide binding site in a membrane preparation isolated from the free-living nematode, *Caenorhabditis elegans*. [^{3}H] Paraherquamide bound specifically to *C. elegans* membranes with an apparent dissociation constant, K_d, of 263 nM. A series of paraherquamide analogs were examined, and their relative affinity for the paraherquamide binding site correlated with their nematocidal activity. Phenothiazines were the only other class of anthelmintics tested which inhibited specific [^{3}H]paraherquamide binding. These results suggest that the anthelmintic activity of paraherquamide and phenothiazine is mediated via an interaction with a common binding site.

Paraherquamide§ is an indole alkaloid first isolated from *Penicillium paraherquei* [1]. Recently, paraherquamide was identified as a potent nematocidal agent [2] with anthelmintic activity against immature *Trichostrongylus colubriformis* in a rodent model system [3]. Paraherquamide is highly efficacious in sheep as a single oral dose against *Haemonchus contortus, Ostertagia circumcincta, T. colubriformis* and *Cooperia curticei* [4]. Ivermectin-resistant and thiabendzole-resistant nematodes remain sensitive to paraherquamide treatment *in vivo* [4], suggesting a novel mode of action for paraherquamide.

Caenorhabditis elegans is a free-living nematode widely used for *in vivo* evaluation of anthelmintic agents [5, 6]. Paraherquamide and a series of naturally occurring analogs are potent nematocidal agents when evaluated in a *C. elegans* motility assay, and in this report we describe a specific [^{3}H]-paraherquamide binding site associated with *C. elegans* membranes and discuss a possible mode of action.

MATERIALS AND METHODS

Materials. Isolation and identification of paraherquamide and its naturally occurring analogs [2] as well as the synthesis of the other paraherquamide analogs have been described previously [7–10]. [^{3}H]-Paraherquamide (C-24: sp. act. 3.54 Ci/mmol) was synthesized by treating a tetrahydrofuran solution of paraherquamide with potassium hydride followed by excess *tert*-butyllithium, quenched with excess ^3H_2O and extracted. The paraherquamide was then purified by preparative thin-layer chromatography using a solvent system of CH_2Cl_2:MeOH (30:2) and verified by TLC and HPLC [11]. Phenothiazine, cambendazole, pyrantel and levamisole were provided by Dr. J. Egerton (Merck, Inc., Rahway, NJ). Ivermectin (22,23-dihydroavermectin B_{1a}) was obtained from Dr. H. Mrozik (Merck, Inc.). All other compounds were obtained from commercial sources.

Membrane preparation. C. elegans, N2 strain was cultivated on NG agar plates covered with a lawn of *Escherichia coli* as previously described [12]. Worms (all stages) were washed off the plates with 25 mM HEPES buffer, adjusted to pH 7.1 with potassium hydroxide. The worms were washed once for 2 min at 1,000 *g*, resuspended in HEPES buffer (approximately 20,000 worms/mL) and then broken up by homogenization in a Braun Homogenizer (Ace Scientific, New Brunswick, NJ) using 0.5 mm glass beads for 30 sec. The homogenate was centifuged for 5 min at 1,000 *g* and the supernatant was centrifuged for 20 min at 28,000 *g*. The resulting pellet was resuspended in HEPES buffer and washed three more times by centrifugation at 28,000 *g* for 20 min in order to dilute cytoplasmic contaminants as well as possible. The final pellet was resuspended in 25 mM HEPES buffer containing 0.1 mM EDTA and used immediately.

Paraherquamide binding. C. elegans membranes were incubated with [^{3}H]paraherquamide at 22° for 20 min in the presence (non-specific binding) or absence (total binding) of a 250-fold molar

† Corresponding author: Dr. James M. Schaeffer, Department of Biochemical Parasitology, R80T-126, Merck Sharp & Dohme Research Laboratories, P.O. Box 2000, Rahway, NJ 07065. Tel. (908) 594-4078; FAX (908) 594-5700.

§ Abbreviations: paraherquamide, spiro[4*H*,8*H*-[1,4] dioxepino[2,3-*g*]indole-8, 7′(8′H)-[5*H*, 6*H*-5a,9a] (iminomethano) [1*H*]cyclopent [f] indolizine]-9,10′(10*H*)dione, 2′, 3′, 8′a, 9′-tetrahydro-1′hydroxy-1′,4,4,8′,8′,11′hexamethyl-(1′α, 5′$\alpha\beta$, 7′β,8′$\alpha\beta$, 9′$\alpha\beta$)-(−); HEPES, 4(2-hydroxyethyl)-1-piperazineethane sulfonic acid; and W-7, *N*-(6-amino-hexyl) -5- chloro-1-naphthalenesulfonamide hydrochloride.

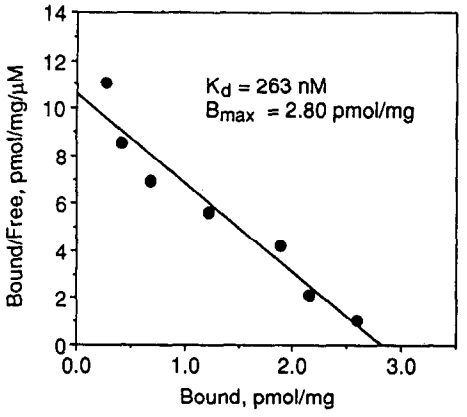


Fig. 1. Scatchard plot analysis for [^{3}H]paraherquamide binding to *C. elegans* membranes. Each data point is the average of four determinations. The SEM was less than 15%, and the experiment was repeated three times with similar results.

excess of unlabeled paraherquamide in glass tubes (13 × 100 mm). The incubation was terminated by rapid filtration over Whatman GF/B filters and rinsed with 15 mL (3 × 5 mL) of ice-cold HEPES buffer. The filters were placed into glass vials containing 10 mL Aquasol II (New England Nuclear, Boston, MA), and the radioactivity was determined by liquid scintillation spectrometry at 62% efficiency. At saturating concentrations of [^{3}H]paraherquamide, the nonspecific binding represented approximately 40% of the total counts. Specific binding was determined by subtracting non-specific from total binding.

The binding data were evaluated using the non-linear curve fitting program LIGAND [13]. This program is based on the first-order mass action law for multiple binding sites and provides a correction for free ligand concentration and non-specific binding. Binding data from the displacement studies were evaluated by the simultaneous fitting of several independent experiments.

Motility assay. Worms were rinsed off the agar plates with HEPES buffer at 22°, washed two times by centrifugation at 1,000 *g* for 2 min, and then resuspended in 25 mM HEPES buffer. Aliquots of the worms (50 μL, approximately 100 worms) were placed into 13 × 100 mm glass test tubes. The compounds to be tested were prepared in dimethyl sulfoxide and added to the worms in a final volume of 500 μL containing 1% dimethyl sulfoxide. After 16 hr of incubation at 22°, the number of worms still motile was determined by examination with a low power dissecting microscope. Greater than 90% of

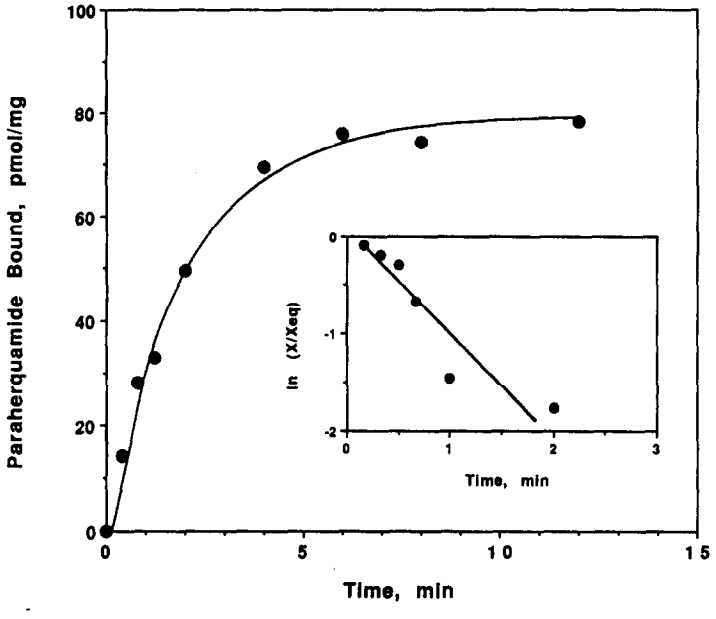
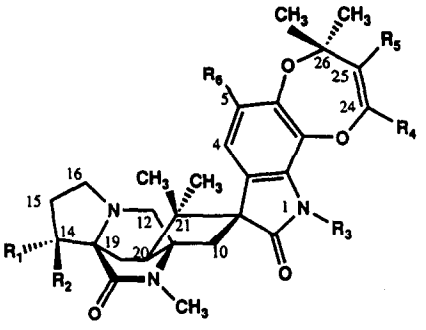


Fig. 2. Rates of association and dissociation of specific [^{3}H]paraherquamide binding to *C. elegans* membranes. Specific binding was measured as a function of time by incubating *C. elegans* membranes with 100 nM [^{3}H]paraherquamide at 22° as described in the text. The rate constant of association, k_1, was derived by dividing the initial slope of the binding by the concentration of [^{3}H]paraherquamide and the concentration of binding sites. Each point is the average of three determinations from a single membrane preparation. Replicate experiments gave similar results. The rate of dissociation was determined by adding a 100-fold molar excess of unlabeled paraherquamide at 20 min after initiation of the incubation and measuring the displacement of [^{3}H]paraherquamide from the membranes. A logarithmic analysis of the data is shown in the inset; the Y-axis represents ln[(binding at time ×)/(binding at time zero)]. The negative slope represents the dissociation rate constant, k_{-1}.

Table 1. Potencies of paraherquamide (1) and structural analogs in the *C. elegans* motility assay and [^{3}H]paraherquamide binding assay



(1) Paraherquamide

Compound No.	R_1	R_2	R_3	R_4	R_5	R_6	K_i* (μM)	ED_{50}† (μg/mL)
1	OH	CH_3	H	H	H	H	0.26	2.5
2	OH	H	H	H	H	H	9.4	60
3	OH	CH_2CH_3	H	H	H	H	0.35	0.75
4	OH	$CH{=}CH_2$	H	H	H	H	0.97	5
5	H	OH	H	H	H	H	>30	>200
6	CH_3	OH	H	H	H	H	22	120
7	CH_2CH_3	OH	H	H	H	H	3.0	30
8	$CH_2C_6H_5$	OH	H	H	H	H	>30	>200
9	H	H	H	H	H	H	10.6	100
10	H	CH_3	H	H	H	H	1.4	6
11	$=CH_2$		H	H	H	H	6.7	40
12	$OSi(CH_3)_3$	CH_3	H	H	H	H	0.30	3
13	OCH_3	CH_3	H	H	H	H	5.5	70
14	O-benzyl	CH_3	H	H	H	H	7.6	65
15	OCH_2CH_3	CH_3	H	H	H	H	0.74	2
16	$OCH_2CH{=}CH_2$	CH_3	H	H	H	H	2.5	15
17	OH	CH_3	CH_3	H	H	H	18	120
18	OH	CH_3	$CH_2CH{=}CH_2$	H	H	H	>30	>200
19	OH	CH_3	H	H_2	H_2	H	>30	>200
20	OH	CH_3	H	H	H	Br	>30	>200

* K_i values are the means of three determinations with the SEM less than 15%.

† ED_{50} values were determined as described in Materials and Methods. Values are the means of four determinations.

the worms continued to swim vigorously in the control tube. The EC_{50} values represent the concentration of drug which immotilizes 50% of the worms.

Protein determination. Protein concentrations were determined by the dye staining technique of Bradford [14] using bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Equilibrium binding parameters. Specific [^{3}H]-paraherquamide binding to *C. elegans* membranes increased linearly as a function of the tissue protein concentration between 0.2 and 4.4 mg/mL of protein. The optimal temperature for [^{3}H]paraherquamide binding was between 18 and 24°. At 22°, the optimal pH for [^{3}H]paraherquamide binding was 6.9, although there was a very broad range of maximal binding between 6.5 and 7.3. Metabolism of [^{3}H]-paraherquamide during the 20-min incubation period was assessed by ethanol extraction of the ligand followed by thin-layer chromatography, and no detectable metabolism of [^{3}H]paraherquamide was observed.

Specific binding of [^{3}H]paraherquamide was saturable with increasing concentrations of [^{3}H]-paraherquamide. The Scatchard plot analysis of these data suggests the presence of a single high-affinity binding site (Fig. 1). The dissociation constant, K_d, was calculated to be 263 nM and the receptor concentration 2.80 pmol/mg protein.

Binding kinetics. At 22°, specific [^{3}H]para-herquamide binding to *C. elegans* membranes was half-maximal at 1.5 min and plateaued at 4.5 min (Fig. 2). The second-order rate constant of

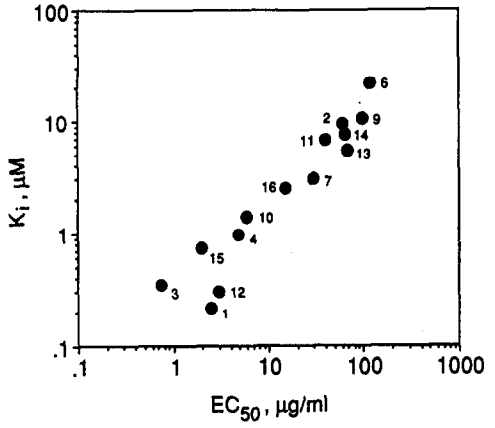


Fig. 3. Correlation between the nematocidal activity (EC_{50} values) and inhibition constants (K_i) determined for 14 paraherquamide analogs. The number associated with each data point refers to the compounds described in Table 1.

association, k_1, was 0.0041 nM^{-1} min^{-1}. The rate of dissociation of the paraherquamide-binding site complex, k_{-1}, was examined by incubating the membranes in the presence of 100 nM [^{3}H]-paraherquamide for 20 min at 22°, and then adding a 100-fold molar excess of unlabeled paraherquamide. The rate of decline of [^{3}H]paraherquamide bound was measured at various times (Fig. 2, inset). The half-life of the paraherquamide-binding site complex was about 0.5 min. The rate constant for dissociation, k_{-1}, was 1.1 min^{-1}. The dissociation constant, k_{-1}/k_1, was calculated to be 268 nM, in close agreement with the value of 263 nM obtained from the equilibrium studies.

Specificity of [^{3}H]*paraherquamide binding.* To assess the specificity of [^{3}H]paraherquamide binding, the ability of various structural analogs to displace [^{3}H]paraherquamide binding was examined (Table 1). More than 100 paraherquamide analogs have been synthesized; however, most of these compounds have greatly reduced nematocidal activity [7–10]. Paraherquamide analogs with modifications at the C-14 position were generally the most biologically active compounds; consequently a series of C-14 substituted analogs were evaluated in the [^{3}H]-paraherquamide binding assay (compounds **2–16**). As shown in Table 1, compounds **3**, **4**, **10**, **12** and **15** were the most potent inhibitors of specific [^{3}H]-paraherquamide binding (K_i values 0.30 to 1.4 μM), although none of the analogs was as active as the parent compound, **1** (K_i = 0.26 μM). Even minor modifications at other portions of the molecule resulted in a nearly total loss of biological activity and a concomitant loss of affinity for the membrane-associated binding site. For example, the addition of a methyl or allyl group at the 1-N position (compounds **17** and **18**, respectively), the reduction at C-24,25 (compound **19**) or the addition of a bromine at the C-5 position (compound **20**) resulted

Table 2. Inhibition of [^{3}H]paraherquamide binding and nematocidal activity of various nematocidal compounds, phenothiazines and dopaminergic compounds

Compound	[^{3}H]Paraherquamide binding (K_i, μM)	Motility assay (EC_{50}, μg/mL)
Anthelmintics		
Levamisole	>1,000	4.5
Ivermectin	>1*	0.005
Cambendiazole	>1,000	1.20
Pyrantel	>1,000	8.5
Phenothiazine	73	35
Phenothiazine analogs		
Chlorpromazine	7	1
Trifluoperazine	21	7
Promethazine	9	3
Perphenazine	9.5	3
Ethopropazine	10.5	2
Dopaminergic agonists/antagonists		
W-7	>250	>200
Dopamine	>1,000	>200
Haloperidol	>1,000	>200
(+)-Butaclamol	>1,000	>200
Spiroperidol	>1,000	>200

K_i values of paraherquamide binding to *C. elegans* membranes were calculated from the respective IC_{50} values using the formula $K_i = IC_{50}/(1 + c/K_d)$, in which c is the concentration of [^{3}H]paraherquamide and K_d is the dissociation constant of [^{3}H]-paraherquamide. The EC_{50} values are the concentrations at which 50% of the worms were immotile. Each value is the average of three independent experiments.

* Ivermectin was not tested at concentrations greater than 1 μM due to the insolubility of the compound in aqueous solutions.

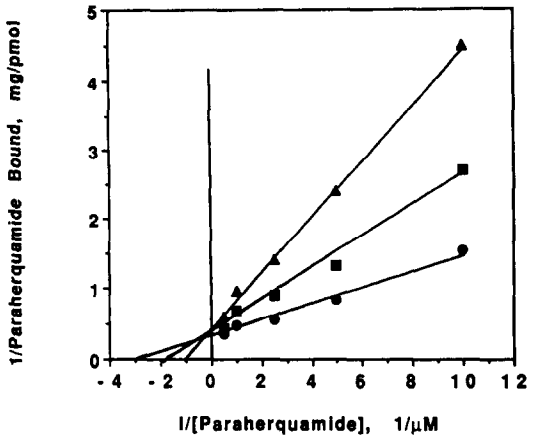


Fig. 4. Lineweaver–Burk analysis of the inhibition of specific binding of [^{3}H]paraherquamide in the absence (●) or presence of phenothiazine (■, 75 μM) or chlorpromazine (▲, 25 μM). The lines were plotted using linear regression analysis. Each point is the mean of three determinations with the SEM less than 15%. The inhibition constants were calculated using the formula:

$$\text{Y-intercept} = \frac{1/K_d(1 + [I])}{K_i}.$$

in compounds with greatly diminished activity in both the binding and motility assays. The correlation coefficient between the inhibition constant for the binding assay and the EC_{50} value in the motility assay was calculated to be greater than 0.95 (Fig. 3), suggesting that the interaction of paraherquamide with its specific binding site is directly related to its nematocidal effect.

Phenothiazine inhibition of [^{3}H]*paraherquamide binding.* To determine whether the paraherquamide binding site is distinct from the binding sites for other anthelmintic agents, specific [^{3}H]paraherquamide binding was quantitated in the presence of high concentrations of structurally unrelated anthelmintics (ivermectin, pyrantel, levamisole, phenothiazine and cambendazole). As shown in Table 2, all of the compounds examined were nematocidal (EC_{50} values 0.005 to 35 μg/mL); however, only phenothiazine competed with [^{3}H]paraherquamide (K_i = 73 μM). A series of phenothiazine derivatives was tested, and all were more potent than the parent compound in the motility assay (EC_{50} = 7–21 μM) and as inhibitors of paraherquamide binding (K_i = 1–7 μM). The effects of phenothiazine and chlorpromazine on [^{3}H]paraherquamide binding are shown in Fig. 4, and the apparent inhibition constants were calculated to be 59 and 10.5 μM, respectively. The maximal concentration of [^{3}H]paraherquamide binding, 275 pmol/mg of protein, was unaltered by the presence of either phenothiazine or chlorpromazine, demonstrating that phenothiazines are competitive inhibitors of [^{3}H]paraherquamide binding.

Phenothiazine has both anthelmintic [15] and antiprotozoal [16] activities; however, the mode of action is not known. The antipsychotic activity of various phenothiazine analogs has been related to their antagonist effect on the dopamine D-2 receptors in mammalian brain tissue [17–19]. It has also been suggested that some phenothiazines act via their modulation of calmodulin activity [20]. Consequently several dopaminergic compounds (dopamine, haloperidol, (+)-butaclamol and spiroperidol) and a calmodulin inhibitor (W-7) were also examined as potential inhibitors of paraherquamide binding. None of these compounds inhibited paraherquamide binding (Table 2).

In summary, the free-living nematode *C. elegans* has specific, high-affinity paraherquamide binding sites. The correlation between the relative binding affinities and nematocidal activities of the various paraherquamide analogs strongly supports the hypothesis that the biological activity is mediated via a ligand–receptor interaction. Phenothiazines inhibited paraherquamide binding, suggesting that both classes of compounds may interact with a common or related binding site. Furthermore, the paraherquamide binding assay provides a rapid and reliable method to evaluate large numbers of paraherquamide analogs.

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