COCHLIOQUINONE A, A NEMATOCIDAL AGENT WHICH COMPETES FOR SPECIFIC [^{3}H]IVERMECTIN BINDING SITES

JAMES M. SCHAEFFER, EASTER G. FRAZIER, ALAN R. BERGSTROM, JOANNE M. WILLIAMSON[†], JERROLD M. LIESCH[††] and MICHAEL A. GOETZ[††]

Departments of Biochemical Parasitology,
[†]Fermentation Microbiology,
[††]Natural Products Chemistry,
Merck Sharp & Dohme Research Laboratories,
P.O. Box 2000, Rahway, New Jersey 07065, U.S.A.

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Cochlioquinone A, isolated from the fungus *Helminthosporium sativum*, was found to have nematocidal activity. Cochlioquinone A is a competitive inhibitor of specific [^{3}H]ivermectin binding suggesting that cochlioquinone A and ivermectin interact with the same membrane receptor.

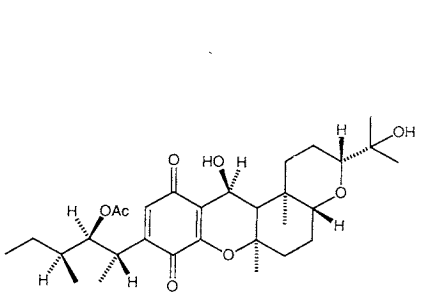
Caenorhabditis elegans is a free-living nematode widely used for the evaluation of anthelmintic agents.[1,2] Using this assay, a nematocidal agent was isolated from a fermentation of *Helminthosporium sativum* and identified as cochlioquinone A. Cochlioquinone A is a yellow pigment originally isolated from a strain of *Cochliobolus miyabeanus*, a parasitic mould of rice.[3] Furthermore, cochlioquinone A is a competitive inhibitor of [^{3}H]ivermectin binding to *C. elegans* membranes suggesting that cochlioquinone A and ivermectin may have a common mode of action.

Materials and Methods

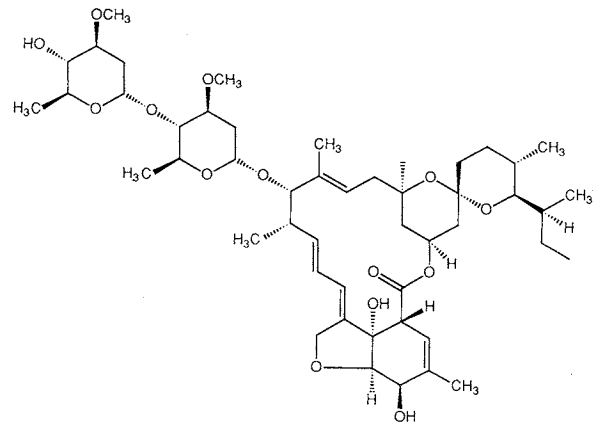
Materials

Ivermectin (22,23-dihydroavermectin B_{1a}) and paraherquamide were supplied by Drs. H. MROZIK and M. FISHER, Merck Sharp & Dohme Research Laboratories (Rahway, N.J.). Cambendazole and levamisole were provided by Dr. J. EGERTON, Merck Sharp & Dohme Research Laboratories. Ivermectin-resistant mutants of *C. elegans* were obtained from D. CULLY, Merck Sharp & Dohme Research Laboratories.

Fig. 1. Structures of cochlioquinone A and ivermectin.



Cochlioquinone A



Ivermectin

[^{3}H]Ivermectin was labeled at the 22,23-position by catalytic hydrogenation with tritium gas to a specific activity of 51.9 Ci/mmol. Purity of [^{3}H]ivermectin was confirmed using TLC on Silica gel 60-F 254 (E.M. Laboratories, Inc.). The plate was developed with chloroform - ethyl acetate - methanol - methylene chloride (9:9:1:2). The Rf value was 0.51, and the [^{3}H]ivermectin was found to be greater than 95% pure. Corn steep liquor was obtained from Sigma Chem. Corp. (St. Louis, MO.); tomato paste was from HUNT's; oat flour was purchased from a retail grocery store; NZ-Amine A was from Sheffield and all other compounds were obtained from commercial sources.

Fermentation Conditions

The seed medium contained (in g/liter distilled water): Corn steep liquor (5.0), tomato paste (40.0), oat flour (10.0), glucose (10.0), $FeSO_4 \cdot 7H_2O$ (0.01), $MnSO_4 \cdot H_2O$ (0.01), $CuCl_2 \cdot H_2O$ (0.00025), $CaCl_2$ (0.001), H_3BO_3 (0.00056), $(NH_4)_2Mo_7O_{24} \cdot 4H_2O$ (0.00019), and $ZnSO_4 \cdot 7H_2O$ (0.002). The pH was adjusted to 6.8 prior to sterilization. The medium was dispensed at 54-ml/250-ml plain Erlenmeyer flask; cotton closures were used. It was sterilized at 121℃ for 20 minutes. The production medium contained (in g/liter distilled water): Glucose (150.0), urea (4.0), NZ-Amine A (4.0), K_2HPO_4 (0.5), $MgSO_4 \cdot 7H_2O$ (0.25), KCl (0.25), $ZnSO_4 \cdot 7H_2O$ (0.9), and $CaCO_3$ (16.5). No pH adjustment was made prior to sterilization. The medium was dispensed at 250-ml/500-ml Erlenmeyer flask; cotton closures were used. It was sterilized at 121℃ for 15 minutes.

Seed cultures of *H. sativum* were inoculated with a source of the culture (three 5 mm agar plugs from a well-sporulated potato - glucose agar plate) and grown on a gyratory shaker (220 rpm; 5.1-cm throw) for 48 hours at 25℃. The culture grew as a mycelial mass. In order to break up the mass and facilitate inoculation of the production medium, approximately 20 small, sterile, ceramic balls were added to the seed flask and it was incubated on a gyratory shaker for 20 minutes.

A portion of the seed culture (12 ml) was used to inoculate each flask containing 250 ml of the liquid production medium. The inoculated medium was added to a 2-liter Erlenmeyer flask which contained vermiculite (70 g; sterilized for 1 hour at 121℃). The mixture was shaken vigorously by hand to coat the vermiculite with the medium. Production flasks were incubated statically at 25℃ for 14 days.

Isolation and Identification of Cochlioquinone A

Bioassay-directed fractionation of a methylethylketone extract of the fermentation broth of *H. sativum* proceeded *via* solvent partitioning (activity was soluble in methylene chloride) followed by two successive silica gel column chromatographic steps with methylene chloride - ethyl acetate gradients. Final purification was achieved by semi-preparative HPLC on a Rainin Dynamax 60A C_{18} column (2.5 cm × 25 cm) eluted at 10 ml/minute with 50% aqueous acetonitrile. The biactive material, whose homogeneity was verified by TLC in several solvent systems and by HPLC (Whatman Partisil-5 ODS- 3, 55% aqueous acetonitrile) was readily identified as the known fungal metabolite cochlioquinone A, by comparison of spectroscopic data (UV, NMR and MS) with published values.[3]

Some instability of the bioactivity was noted during the course of purification; this was ultimately traced to the sensitivity of cochlioquinone A to light, which is particularly pronounced in methanolic solutions.

Motility Assay

Worms were rinsed off the agar plates with room temperature 5 mM Trizma base, adjusted to pH 7.5 with HCl, washed two times by centrifugation at 1,000 × *g* for 2 minutes and then resuspended in the 5-mM Tris 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 added to the worms in a final volume of 500 μl of the Tris buffer containing 1% dimethyl sulfoxide. After 16 hours incubation at 22℃, the number of worms still motile was determined by examination with a low power dissecting microscope. Greater than 90% of the worms continued to swim vigorously in the control tube.

Membrane Preparation

C. elegans, N2 strain was cultivated on NG agar plates covered with a lawn of *Escherichia coli* as previously described.[4] Worms (all stages) were washed off the plates with the 5-mM Tris buffer. The worms

were centrifuged 2 minutes at 1,000×*g*, resuspended in buffer (approximately 20,000 worms/ml) and then broken up by homogenization in a Braun Homogenizer (Ace Scientific, New Brunswick, N.J.) using 0.5 mm glass beads for 20 seconds. The homogenate was centrifuged 2 minutes at 1,000×*g* and the supernatant centrifuged 20 minutes at 28,000×*g*. The resulting pellet was resuspended in buffer and washed three more times by centrifugation at 28,000×*g* for 20 minutes in order to dilute cytoplasmic contaminants as well as possible. The final pellet was resuspended in Tris buffer and used immediately.

Ivermectin Binding Assay

The membrane preparations (1.0 ml in 13×100 mm glass tubes) were incubated with [^{3}H]ivermectin at 22℃ for 45 minutes in the presence (nonspecific binding) or absence (total binding) of a 500-fold molar excess of unlabeled ivermectin. The incubation was terminated by rapid filtration over Whatman GF/B filters (pretreated with 0.15% polyethylimine and 0.25% Triton X-100 in order to minimize nonspecific binding) and rinsed with 15 ml (3×5 ml) of ice-cold HEPES buffer containing 0.5% Triton X-100. The filters were placed into glass vials containing 10 ml Aquasol II (New England Nuclear, Boston, MS), and radioactivity was determined by liquid scintillation spectometry at 62% efficiency. Specific binding was calculated by subtracting nonspecific total binding.

Protein Assays

Protein concentrations were determined using the dye staining technique of BRADFORD.[5]

Results

Cochlioquinone A Effect on *C. elegans* Motility

C. elegans was incubated in the presence of increasing concentrations of cochlioquinone A and the percentage of motile worms quantitated after 16 hours. The ED_{50} (the concentration at which 50% of the worms remain motile) for cochlioquinone A was 135 μM (Fig. 2). Ivermectin-resistant mutants of *C. elegans* were also less sensitive to cochlioquinone A, with an ED_{50} of greater than 1 mM. In parallel experiments, the

Fig. 2. Cochlioquinone A inhibition of *Caenorhabditis elegans* motility.

Wild type *C. elegans* were maintained in the presence of increasing concentrations of cochlioquinone A (●), or ivermectin (○) and after 16 hours the percentage of motile worms was determined. Parallel experiments were conducted with ivermectin resistant mutants of *C. elegans* incubated with cochlioquinone A (■) or ivermectin (□). This experiment was replicated three times with similar results.

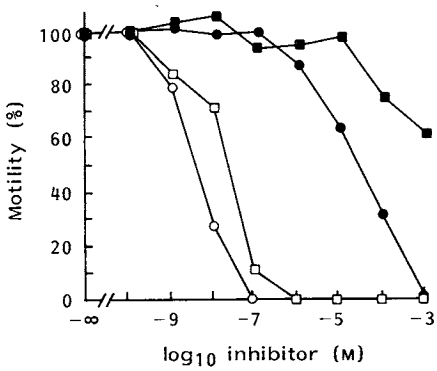
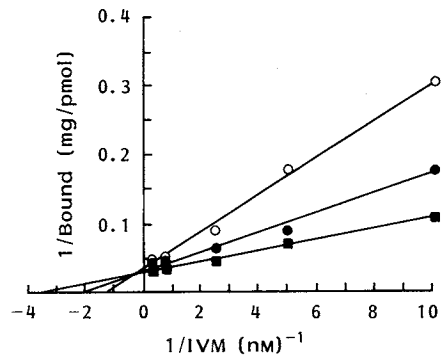


Fig. 3. Lineweaver-Burke analysis of the inhibition of specific ivermectin binding by cochlioquinone A.

Specific ivermectin binding was determined by incubating *Caenorhabditis elegans* membranes with increasing concentrations of [^{3}H]ivermectin as described in the text in the absence (■) or presence of cochlioquinone A (45 μM, ● and 90 μM, ○).



The lines were plotted using linear regression analysis. Each point represents the mean of four determinations; standard error of the mean values were less than 10%. The inhibition constants were calculated using the formula y-intercept = $-1/Kd$ $(1+[1]/Ki)$.

ED_{50} values for ivermectin using wild type and ivermectin resistant *C. elegans* were 5 and 75 nM, respectively.

Cochlioquinone Inhibition of Ivermectin Binding

In order to determine whether cochlioquinone A is interacting directly with the ivermectin binding site, *C. elegans* membranes were incubated with increasing concentrations of [^{3}H]ivermectin in the presence or absence of a fixed concentration of cochlioquinone A (45 and 90 μM). The double reciprocal plot of these data (Fig. 3) yield a series of lines intersecting at the y-axis, indicating that cochlioquinone A is a competitive inhibitor of ivermectin binding to sites in *C. elegans* membranes with an inhibition constant, *Ki*, of 30 μM. The affinity of [^{3}H]ivermectin binding to *C. elegans* membranes in the absence of cochlioquinone is 0.24 nM. Other anthelmintic agents (cambendazole, phenothiazine, levamisole and paraherquamide) were tested and none inhibited ivermectin binding at concentrations up to 1 mM (data not shown).

Discussion

The mode of action of ivermectin (and all of the biologically active avermectin analogs) is thought to be mediated *via* a specific receptor site regulating a non-GABA gated chloride ion channel.[6,7] In invertebrate neuromuscular preparations, subnanomolar concentrations of ivermectin significantly increase the membrane permeability to chloride ions. We have previously identified and characterized a specific ivermectin binding site in a membrane fraction prepared from the free living nematode *C. elegans*.[2] The results presented in this study demonstrate that cochlioquinone A is a competitive inhibitor of ivermectin binding with an inhibition constant, *Ki*, of 30 μM. In addition, cochlioquinone A has nematocidal activity as determined in a motility assay (ED_{50}=135 μM). Although there is no obvious structural similarity between cochlioquinone A and the avermectins, the inability of cochlioquinone A to immotilize the ivermectin-resistant *C. elegans* mutants further supports the hypothesis that cochlioquinone A and ivermectin are acting at the same binding site. We are currently studying the effect of cochlioquinone on ivermectin-sensitive chloride channels. This is the first report of a non-avermectin interacting with an invertebrate avermectin binding site, and although the affinity of cochlioquinone A for the ivermectin binding site is relatively low, cochlioquinone A may provide a useful tool for studying the anthelmintic activity of the avermectins.

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