INHIBITION OF CHITIN METABOLISM BY AVERMECTIN IN SUSCEPTIBLE ORGANISMS

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Avermectin inhibits *Mucor miehei* and *Artemia salina* chitin synthesis and to a degree DNA synthesis in the former. The antibiotic interferes with chitin turnover in brine shrimp and inhibits *Streptomyces antibioticus* chitinase activity *in vitro*. In light of the proposed mode of action of avermectin and the anomolies in the literature, it is proposed that avermectin can kill susceptible organisms not only by a neurotoxic mechanism but also by inhibiting chitin turnover and synthesis at low concentration and thus the molting/ecdysis process.

Avermectin is a macrocyclic lactone antibiotic produced during the fermentation of *Streptomyces avermitilis*[1,2]. This antibiotic is a potent acaricide/insecticide/anthelmintic agent[3] whose mode of action against susceptible organisms is proposed to be as a neurotoxin[4]. Avermectin has been shown to interfere with γ-aminobutyric acid (GABA) binding to synaptic receptors thus disturbing Cl^- passage through the end plate which results in failure of nervous impulse passage and paralysis[4,5,6].

This study describes some exploratory experiments on two avermectin susceptible organisms, *Artemia salina,* the brine shrimp and *Mucor miehei,* a fungus (the latter contains no nervous system or associated GABA receptors) to determine whether avermectin affects other cellular processes.

Materials and Methods

Avermectin Production

Avermectin was produced by the fermentation of *S. avermitilis* (ATCC 31272) in M8 medium (g/liter; starch 20, glucose 10, $CaCO_3$ 3, Bacto-Casitone 2, N-Z amine A 2, yeast extract 2 and beef extract 2). Routinely organisms were grown on M8 agar slants (with 2% Bacto Agar) for 4~7 days at 28°C before transfer to 150 ml M8 in 500 ml conical flasks. These cultures were grown at 28°C for 4 days with vigorous shaking before transfer of a 10% inoculum to fresh M8 medium and further incubation. After 4~6 days, cultures were harvested by centrifugation at room temperature. The recovered cells were extracted with methanol for 30 minutes at room temperature. The methanol extract was recovered from the cell debris and concentrated before storage at −20°C. This extract, containing avermectin, was used in all experiments reported. Avermectin was quantified by a high performance liquid chromatography technique similar to that described by others[2]. Avermectin purified by this HPLC technique was used in certain critical experiments and gave comparable responses to the crude material (data not shown).

Biological Test Organisms

Brine shrimp (*A. salina*) were hatched from egg stage at 25°C in a synthetic marine environment (Instant Ocean, Aquarium System Inc., Mentor, OH, USA). 48 hours nauplii were used in all bioassays reported. *M. miehei* (ATCC 16457) was grown aerobically to stationary phase at 30°C in 150

ml portions of modified Sabouraud medium with glucose (Difco Inc., Detroit, MI, USA) in 500 ml conical flasks (5~7 days).

Bioassays

Avermectin and other compounds were subjected to an agar dilution (2-fold) antimicrobial toxicity screen which included both Gram-positive and negative aerobes and anaerobes and fungi. Compounds were also assayed for toxicity towards brine shrimp. Routinely, samples of compounds were added to 100 ml portions of synthetic seawater, after which between 10 and 80 nauplii were added. The samples were incubated at room temperature (25°C) for up to 72 hours after which mortality was recorded. LC_{90} were recorded as the minimal concentration of compound that killed at least 90% of the nauplii after an appropriate time. In all experiments untreated controls were exposed to the solvent used to solubilize the avermectin; in all cases the concentration of methanol never exceeded 0.5% which was non-toxic.

Macromolecular Synthesis and Active Transport Assays

Rates of active transport of amino acids and of synthesis of DNA, RNA, protein and chitin were determined as described elsewhere[7,8] by measuring uptake of an amino acid mixture, and incorporation of thymidine, uracil, amino acid mixture and *N*-acetylglucosamine (all ^{3}H-labeled) into TCA-insoluble material. In these experiments, the test organisms, *M. miehei* (1~2 mg dry weight/ml) and *A. salina* (100~1,000 nauplii/ml), were added to reaction mixtures of 50 mM sodium phosphate (pH 7.4) and synthetic seawater, respectively. Organisms were incubated with potential inhibitor at an appropriate concentration for 20~30 minutes before addition of radioactive compound. In all experiments, untreated controls were exposed to equal concentrations of the solvent for inhibitor.

Chitin Turnover

Chitin turnover in brine shrimp was determined by a pulse-chase technique. Routinely, approximately 10^5 brine shrimp nauplii in 50 ml of synthetic seawater were incubated with 100 μCi [^{3}H]-*N*-acetylglucosamine (20~40 Ci/mmol) for 1 hour at room temperature to allow for uptake and incorporation of the chitin precursor into TCA-insoluble chitin. After washing the nauplii with 2×50 ml of seawater, unlabeled *N*-acetylglucosamine (1 mM) was added and the shrimp were incubated 30 minutes at room temperature to chase the radiolabel into stable TCA-precipitable material. After washing twice in seawater (50 ml), the shrimp suspension was challenged with avermectin or polyoxin D at appropriate concentrations. Samples (1 ml) were removed at various intervals and the radioactivity incorporated into TCA-insoluble material was determined as described before[7,8].

Chitinase Assay

Chitinase (purified from *Streptomyces antibioticus*) was obtained from Calbiochem (San Diego, CA, USA) and reconstituted in 50 mM sodium phosphate buffer pH 7.4 and stored at −10°C. Chitinase assays were performed in 50 mM sodium acetate - Tris buffer at pH 6.6 at 30°C in a Lambda 3 System spectrophotometer (Perkin-Elmer) on continuous record at 510 nm[9]. The reaction mixture contained 3 ml γ-chitin-red at 1 mg/ml in Tris - acetate buffer, 0.4 ml enzyme at 33 mg/ml in sodium phosphate buffer and potential inhibitor. Methanol, the solvent for potential inhibitors, was always added to all cuvettes to a final concentration of 0.4 ml/3.4 ml volume. This methanol concentration prevented growth of potential contaminating bacterial organisms during the 30~48-hour incubations at 30°C. Since no extinction coefficient for γ-chitin-red (an insoluble substrate) was available, a unit of enzyme activity was defined as that enzyme required to liberate 0.001 absorbance unit of dye from substrate in 1 minute at 30°C.

Chemicals

All chemicals were obtained from Sigma Chemical Company, St. Louis, MO except polyoxin D, chitinase and γ-chitin-red which was obtained from Calbiochem, San Diego, CA, USA and radioisotopes obtained from ICN, Irvine, CA USA and NEN, Boston, MA, USA. Media components were purchased from Difco Company, Detroit, MI, USA and Humko Sheffield, Lynnhurst, NJ, USA.

Results

Antibiotic Sensitivity of Various Bacteria, Fungi and Brine Shrimp

All bacteria and the yeast, *Candida albicans,* were resistant to avermectin while the three fungi, *Aspergillus niger, Piricularia oryzae* and *M. miehei* were susceptible to the antibiotic (Table 1). Brine shrimp were also susceptible to avermectin exhibiting an LC_{90} of 0.8 ng/ml (data not shown).

Effect of Avermectin an Active Transport and Macromolecular Synthesis in *M. miehei* and *A. salina*

Table 2 presents data on the effect of both low and high concentrations of avermectin on the various cell processes in the two susceptible organism. The concentrations chosen were at or near the minimal inhibitory concentration and ten-fold above these values. While most cell processes were relatively unaffected by the antibiotic, DNA synthesis in *M. miehei* and wall synthesis in both organisms were particularly susceptible. To confirm that incorporation of [^{3}H]-*N*-acetylglucosamine into TCA-insoluble material did measure chitin synthesis, we determined the effect of polyoxin D on this activity[10]. Fig. 1 shows that not only did avermectin inhibit incorporation of *N*-acetylglucosamine, but polyoxin D at 100 μg/ml was also an effective chitin synthesis inhibitor in brine shrimp.

Table 1. Minimal inhibitory concentration (MIC) of avermectin against a number of microbes.

Organism	MIC* (μg/ml)
Actinomyces viscosus	>17
Clostridium perfringens	>17
C. septicum	>17
Bacteroides fragilis	>17
B. multiacidus	>17
Streptococcus faecalis	>17
S. mutans	>17
S. bovis	>17
Lactobacillus casei	>17
Fusobacterium necrophorum	>17
Candida albicans	>17
Aspergillus niger	2.1
Mucor miehei	2.1
Piricularia oryzae	17
Bacillus subtilis	>17
Erwinia amylovora	>17
Staphyloccocus aureus	>17
Escherichia coli	>17
Salmonella typhimurium	>17
Pseudomonas aeruginosa	>17
Micrococcus luteus	>17

* The MIC was determined by the agar dilution technique employing 2-fold dilutions.

Fig. 1. Effect of avermectin and polyoxin D on chitin synthesis in brine shrimp.

A. salina was incubated in the absence (▲) or presence of avermectin (A) at 10 ng/ml (□) or 100 ng/ml (■) or polyoxin D (B) at 100 μg/ml (○) for 20 minutes before addition of [^{3}H]-*N*-acetylglucosamine. At various times samples were removed and assayed for incorporation of label into TCA-insoluble material as described in the text.

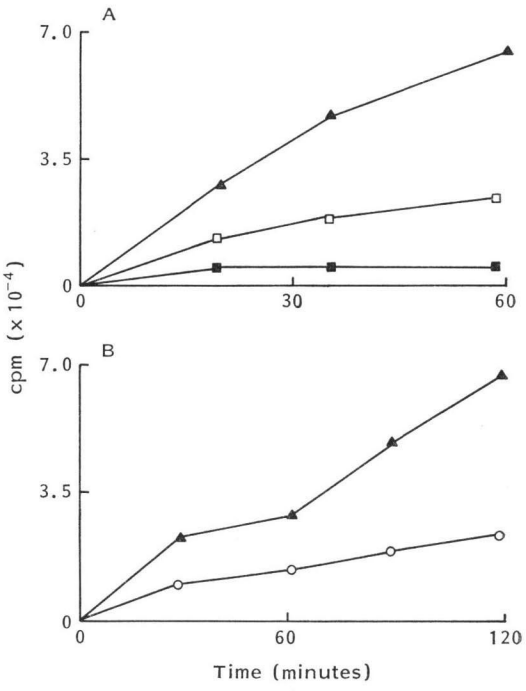


Table 2. Effect of avermectin on biochemical processes in *M. miehei* and *A. salina*.

Cell process*	*M. miehei*		*A. salina*	
	Concentration of avermectin (μg/ml)	% activity of control	Concentration of avermectin (ng/ml)	% activity of control
Active transport	4.5	77	10	70
	45	62	100	70
Protein synthesis	4.5	111	10	137
	45	139	100	83
RNA synthesis	4.5	85	10	81
	45	104	100	38
DNA synthesis	4.5	59	10	113
	45	12	100	101
Chitin synthesis	4.5	43	10	47.3
	45	45	100	7.3

* *M. miehei* and *A. salina* were incubated in the presence of a potential inhibitor for at least 20 minutes before rates of incorporation or transport were determined as in Materials and Methods. Avermectin was dissolved in methanol and at most 25 μl of the solution was added per ml reaction mixture. Controls had equal volumes of methanol.

Chitin Turnover in Brine Shrimp

Since avermectin appeared to interfere with chitin synthesis, it is conceivable that it might interfere with chitin turnover. Fig. 2 shows the effect of avermectin on stability of chitin in brine shrimp. As is evident, avermectin at 10 ng/ml slowed down the rate of liberation of radioactive from TCA-insoluble material *i.e.* slowed down chitin turnover. In separate experiments not shown polyoxin D (100 μg/ml) also slowed down chitin turnover although the addition of both avermectin and polyoxin D did not show a synergistic effect.

Fig. 2. Effect of avermectin on stability of chitin in brine shrimp.

A. salina chitin was labeled with [^{3}H]-*N*-acetylglucosamine as described in the text. Portions of the labeled population were incubated in a synthetic seawater in the absence (□) or presence of 10 ng/ml avermectin (■). Samples were removed at various intervals and assayed for ^{3}H-labeled TCA-insoluble material.

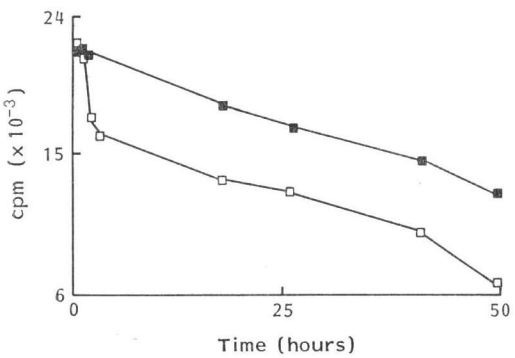
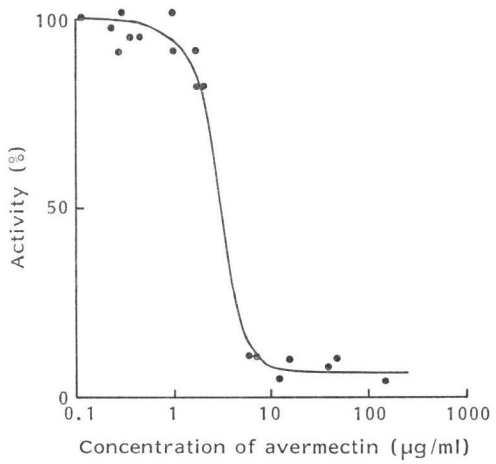


Fig. 3. Effect of avermectin on *Streptomycete* chitinase.

Avermectin at various concentrations was incubated at 30°C with a *Streptomycete* chitinase in the presence of the chromogenic substrate, γ-chitin-red. The absorbance of the suspension was monitored continuously at 510 nm. Enzyme activities were calculated from the linear portion of the curves and were converted to % of control, (enzyme activity recorded for controls were always in the 0.60~0.72 units/cuvette).



Effect of Avermectin on Chitinase

Since avermectin has been shown to inhibit chitin synthetase and chitin turnover, it is conceivable that it might be an effective inhibitor of the enzyme chitinase. Unfortunately, since a source of active chitinase from an invertebrate is not available commercially, we chose to use the enzyme from *S. antibioticus* (Calbiochem) as a model system. Using a colorimetric assay described by others[9], we investigated the effect of avermectin on this enzyme activity. From Fig. 3, it is evident that avermectin was a potent inhibitor and exhibited an apparent K_i of approximately $2 \sim 3$ μg/ml against chitinase.

Discussion

Research workers at Merck have shown convincing evidence that avermectin is capable of binding to rat brain membranes and eliciting, an alteration in the binding of GABA[4]. Using a relatively crude membrane system from rat brain, they have been able to detect a picrotoxin, bicuculline sensitive, Cl^- dependent stimulation of GABA binding to its receptor[4]. This enhancement is associated with an increase in number of receptors but no alteration in their affinity indicating that avermectin exposes more receptors and does not expose a new species. However, these studies employ a test organism, the rat, that is intrinsically resistant to avermectin. Further they propose that this enhancement of GABA binding observed *in vitro* will result in an increase in Cl^- permeability of the membrane resulting in loss of action potential in the neuron, loss of motor function and eventual paralysis *in vivo*[5,6]. This increase in Cl^- permeability and loss of motor function has been seen in studies employing avermectin sensitive organisms, a crustacean, and a nematode[5,6]. In further support of their GABA receptor target model for avermectin toxicity, they cite their studies of various analogues of avermectin. While their conclusions are supported by their data for four of the five analogues of avermectin, the data from the aglycone form of avermectin is at variance (Table 3). This analogue is a relatively effective agent against their model pest (58%), *Caenorhabditis elegans* while it is relatively inactive in their receptor assay (4%) when compared with avermectin. This suggests that while their model may explain some of the data, there are situations where an alternative mode of action is possible.

In this paper, we have described studies where we have examined the sensitivity of an invertebrate, *A. salina,* the brine shrimp, and *M. miehei,* a fungus to avermectin. In both cases, the organisms are avermectin sensitive, yet while the former should contain GABA receptors associated with a nervous system, the latter, containing no nervous system, should possess no neuronal GABA receptors. Thus, it is logical to suppose that avermectin is capable of killing *Mucor,* at least, in another manner. That avermectin inhibited the incorporation of *N*-acetylglucosamine into TCA-insoluble material (chitin) in both organisms would indicate that chitin synthesis could be a target process in both organism. This association was strengthened by the findings that avermectin slowed down chitin turnover in brine shrimp and effectively inhibited chitinase activity in an *in vitro* assay system. Thus, avermectin appears to act as a chitin metabolism inhibitor. Examination of two avermectin analogues, ivermectin and

Table 3. Comparison of properties of avermectin (AVM) and analogues.

Analogue	Activity of analogue (% of AVM)				
	AVM receptor activity	*C. elegans* toxicity	*A. salina* toxicity	Chitin synthesis in *A. salina*	*Streptomyceste* chitinase
AVM	100	100	100	100	100
Dihydro-AVM	40	34	120	150	200
AVM-aglycone or milbemycin	4	58	80	100	100
Octahydro-AVM	2	<1	NT	NT	NT
Dihydro-AVM diacetate	1	<1	NT	NT	NT

NT: Not tested (analogues not available). Data on AVM receptor activity and *C. elegans* toxicity from PONG and WANG[11], other data from this paper and unpublished data.

milbemycin (the aglycone form of avermectin) indicates that the chitin metabolism inhibitor action of avermectin and analogues appears to explain the discrepancies noted in the Merck data (Table 3). Thus, the lack of activity of the aglycone form of avermectin in the receptor assay yet its effective ability to kill the model pest, *C. elegans,* can be accounted for by its strong activity *versus* chitin synthesis, turnover and degradation processes (Table 3).

Table 4. Relationship between taxonomy of organisms, sensitivity to avermectin and presence of GABA receptors and chitin.

Organism	Sensitivity to AVM	GABA receptor present**	Chitin presence**
Mammals			
Rat	−	+*	−
Arthropods			
Insects		+	+
Mites	+	+	+
Crustaceans	+	+	+
Nematodes			
Ascaris	+	+	+
Cestodes			
Flukes	−	−	−
Trematodes			
Tapeworms	−	−	−
Fungi			
Mucor	+	−	+

* Although rat have GABA receptors, they are protected from avermectin by the blood brain barrier[4].

** Chitin presence confirmed by a computer assisted literature search over the years 1968～1983 in both Chemical and Biological Abstracts and the review by LESTAN[12]. GABA receptor presence was ascertained by a similar computer search and publications[4,11].

While it is likely that avermectin interacts directly with the chitinase enzyme or binds to substrate preventing its interaction with the enzyme (the assays were performed *in vitro* using a purified enzyme system), it is not clear whether the effect of the antibiotic on chitin synthesis and turnover is direct or by indirect means such as *via* hormone control. To determine the precise mechanism would require a more detailed *in vitro* study.

Table 4 presents the spectrum of activity of avermectin against a number of organisms ranging from a mammal (rat) to a fungus (*M. miehei*). As can be seen, the specificity observed for avermectin correlates with the presence of GABA receptors in all organisms studied except rat which is avermectin resistant and possesses GABA receptors and the fungus that is avermectin sensitive and does not contain GABA receptors. However, rat GABA receptors are in the central nervous system protected by a blood brain barrier. This table also presents data on the presence of chitin in these organisms. As can be seen, the chitin target model fits the data as well as, if not better than, the GABA receptor target model, particularly in cases of the rat and fungus. Clearly, we believe the chitin metabolism target model is a viable alternative mechanism for the mode of action of avermectin. We feel that at high concentrations avermectin may very well kill susceptible organisms by paralysis but at low levels the action is by disturbance of chitin metabolism perhaps during the ecdysis process.

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