# THE LACK OF ANTIFUNGAL ACTIVITY BY AVERMECTIN B1a

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*Streptomyces avermitilis* produces avermectin, oligomycin and a polyene antifungal. The latter two compounds account for the antifungal activity in the methanol extracts of the fermentation broth. Pure avermectin does not inhibit fungi or affect fungal chitin metabolism.

Avermectin (AVM) was recently reported to inhibit fungi by interfering with chitin metabolism[1]. The evidence for this conclusion was based on data obtained with a methanol extract of *Streptomyces avermitilis* ATCC 31272 culture grown under conditions for avermectin production. A minimal inhibitory concentration was reported against three filamentous fungi. The sample also inhibited *N*-acetylglucosamine incorporation into chitin, reduced chitin turnover in brine shrimp and inhibited chitinase purified from *Streptomyces antibioticus*. These results were not confirmed in our laboratory using an avermectin sample purified by solvent extraction, Sephadex LH-20 and HPLC chromatography. The antifungal activity in the methanol extract was accounted for by oligomycin and a polyene. Pure AVM B1a had no detectable antifungal activity of effect on fungal chitin metabolism. It is concluded that AVM acts specifically as an antihelmintic and insecticide by reducing the membrane potential[2,3].

## Materials and Methods

### Organisms

The fungal strains were obtained from the Merck Culture Collection, the American Type Culture Collection or the Yeast Genetic Stock Center.

### Fungal Cultures

Yeast blastospores were obtained from a 24-hour old shake culture grown at 30°C in Sabouraud dextrose broth. Filamentous fungal spores were obtained by flooding a 2-week sporulated culture on potato - dextrose agar with 0.01% Tween 80. The spores were harvested by aseptically scraping the plate.

### *In Vitro* Testing

The minimal inhibitory concentration (MIC) was determined by serial dilution. The size of the inoculum was $10^5$ blastospores/ml and $10^4$ spores/ml. The fungi were assayed on potato - dextrose agar (PDA), Sabouraud dextrose agar (SDA) or yeast extract - salts (YE-Salts) medium supplemented with 2% glucose or EtOH[4]. The plates were incubated at 28°C for 48 hours and read for visible growth. The MIC data were not affected by incubation for an additional 5 days.

### Incorporation Studies with Growing Cells

Spores from *Rhizomucor miehei* ATCC 16457 were harvested as described. The spores were germinated by inoculating Sabouraud dextrose broth and incubating overnight at 28°C, 250 rpm. The germlings were harvested, washed by Millipore filtration and resuspended in an equal volume of VOGEL's synthetic medium supplemented with 2% maltose. The germlings were incubated at 37°C, 220 rpm for 2.5 hours. After incubation, 5 ml of cells were transferred to tubes containing labeled

precursor. Five minutes later drugs were added. The reaction tubes were incubated with shaking at 37°C and samples were removed at intervals.

The reaction mixture contained 1 mM [1-$^{14}$C]glucosamine (specific activity 0.2 Ci/$\mu$mol). The TCA precipitate was collected on glass fiber filters. The labeled cells were fractionated as described below.

Pulse-chase Studies with Growing Cells

Spores of *R. miehei* ATCC 16457 were harvested and grown as described. The cells were pulsed with [1-$^{14}$C]glucosamine (1 mM at 0.2 $\mu$Ci/$\mu$mol) for 30 minutes. The isotope was chased by collecting the cells on a Millipore filter washing with medium and resuspending in VOGEL's synthetic medium supplemented with 2% maltose and 0.5% glucosamine.

Wall Fractionation

Wall polysaccharides were extracted from glucosamine labeled cells according to the method of MAHADEVAN and TATUM[5]. The cells were extracted with 95% EtOH at 80°C for 15 minutes followed by $CHCl_3$ - MeOH (2: 1) at room temp for 2 hours. The solvent extracted pellet was digested with 1 N NaOH at room temp for 17 hours. The alkali-insoluble pellet was digested with 1 N $H_2SO_4$ at 100°C for 60 minutes. The remaining pellet was treated with 400 $\mu$g/ml of chitinase for 48 hours at 28°C in 0.05 M phosphate at pH 6.9 containing penicillin and chloramphenicol at 200 $\mu$g/ml.

## Results

### Avermectin Purification

During the isolation of the AVM components[6] many impurities were detected and characterized to varying degrees. One fraction from Sephadex LH-20 chromatography yielded crystalline material from methanol - water. This compound was characterized by microanalyses, UV, NMR and MS but since it did not exhibit anthelmintic activity it was not pursued further. Its UV spectrum showed peaks at 226 nm and 232 nm, about 11 nm lower than AVM and its elution time in the HPLC system was very close to that of AVM A2a. This compound interfered with HPLC determination of AVM A2a until the wave-length of the detector was moved up to 246 nm as reported earlier[6]. It was separated from AVM by the first Sephadex LH-20 chromatography step, eluting much later than AVM B components. The compound was later identified as oligomycin[7].

Table 1. Minimal inhibitory concentration (MIC) of avermectin B1a against a number of fungi.

| | MIC ($\mu$g/ml) | | | |
|---|---|---|---|---|
| | PDA | SDA | YE-Salts 2% glucose | YE-Salts 2% EtOH |
| *Saccharomyces cerevisiae* MY 34 | >400 | >400 | >400 | >400 |
| *S. cerevisiae* X2180 | >400 | >400 | >400 | >400 |
| *Candida albicans* MY 992 | >400 | >400 | >400 | >400 |
| *C. albicans* MY 1013 | >400 | >400 | >400 | >400 |
| *C. albicans* MY 1029 | >400 | >400 | >400 | >400 |
| *C. albicans* MY 1055 | >400 | >400 | >400 | >400 |
| *Aspergillus* sp. MF 11 | >400 | >400 | >400 | >400 |
| *Alternaria* sp. MF 3550 | >400 | >400 | >400 | >400 |
| *Phoma* sp. MF 4332 | >400 | >400 | >400 | >400 |
| *Cochliobolus miyabeanus* MF 4626 | >400 | >400 | >400 | >400 |
| *Rhizomucor miehei* ATCC 16457 | >400 | >400 | >400 | >400 |

Agar dilution MIC determined by applying yeast cells at $10^5$ cfu and filamentous fungi at $10^4$ cfu to medicated agar media. The assay plates were incubated at 28°C for 48 hours and read for visible growth. There was no change in plates read at 7 days.

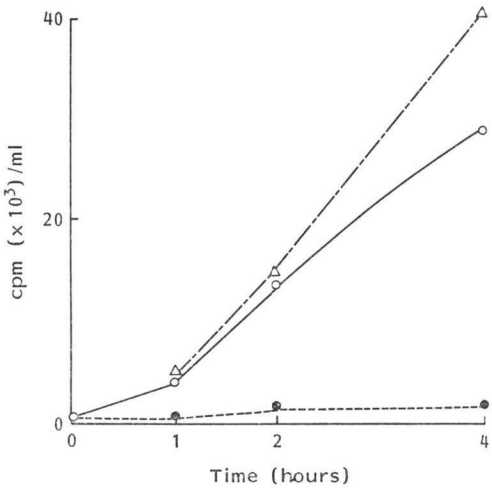
Table 2. Minimal inhibitory concentration (MIC) of oligomycin against a number of fungi.

| | MIC ($\mu$g/ml) | | | |
|---|---|---|---|---|
| | PDA | SDA | YE-Salts 2% glucose | YE-Salts 2% EtOH |
| *Saccharomyces cerevisiae* MY 34 | >400 | >400 | >400 | <0.75 |
| *S. cerevisiae* X2180 | >400 | >400 | >400 | <0.75 |
| *Candida albicans* MY 992 | >400 | >400 | >400 | >400 |
| *C. albicans* MY 1013 | >400 | >400 | >400 | >400 |
| *C. albicans* MY 1029 | >400 | >400 | >400 | >400 |
| *C. albicans* MY 1055 | >400 | >400 | >400 | >400 |
| *Aspergillus* sp. MF 11 | <0.75 | <0.75 | <0.75 | <0.75 |
| *Alternaria* sp. MF 3550 | <0.75 | <0.75 | <0.75 | <0.75 |
| *Phoma* sp. MF 4332 | <0.75 | <0.75 | <0.75 | <0.75 |
| *Cochliobolus miyabeanus* MF 4626 | <0.75 | <0.75 | <0.75 | <0.75 |
| *Rhizomucor miehei* ATCC 16457 | <0.75 | <0.75 | <0.75 | <0.75 |

Agar dilution MIC determined by applying yeast cells at $10^5$ cfu and filamentous fungi at $10^4$ cfu to medicated agar media. The assay plates were incubated at 28°C for 48 hours and read for visible growth. There was no change in plates read at 7 days.

Fig. 1. Incorporation of radioactivity from [1-$^{14}$C]-glucosamine into acid-insoluble fraction of cells of *R. miehei*.

Untreated control cells (○) or cells treated with 100 $\mu$g/ml of avermectin B1a (△) or 1 $\mu$g/ml oligomycin (●) were labeled as described in Materials and Methods. Radioactivity incorporated into acid-insoluble material was evaluated as described.




Another impurity tentatively identified during the AVM isolation work was an antifungal agent of the methylpentaene type[8]. Antifungal activity could be correlated with a UV spectrum which showed peaks at 322, 338 and 355 nm. Since this activity could be completely separated

Table 3. Effect of avermectin B1a on the incorporation of [1-$^{14}$C]glucosamine into the cell wall fractions of *R. miehei*[a].

| Condition | Control | AVM B1a (100 $\mu$g/ml) |
|---|---|---|
| Solvent extract | 41 | 77 |
| Alkali-soluble | 159 | 212 |
| Acid-soluble | 609 | 632 |
| Chitinase sensitive | 7,985 | 6,632 |

[a] *R. miehei* spores were germinated and labeled as described. The cells were labeled for 2 hours during the exposure to AVM B1a. A 5-ml sample was harvested and fractionated as described. Data are expressed as counts per minute per ml.

Table 4. Effect of avermectin B1a on the incorporation of [1-$^{14}$C]glucosamine into the cell wall fractions of *R. miehei*[a].

| Condition | Control | AVM B1a (100 $\mu$g/ml) |
|---|---|---|
| Solvent extract | 32 | 26 |
| Alkali-soluble | 129 | 123 |
| Acid-soluble | 229 | 143 |
| Chitinase sensitive | 1,660 | 2,092 |

[a] *R. miehei* spores were germinated and labeled as described. The cells were labeled for 30 minutes and exposed to AVM B1a during the 2 hours chase. A 5-ml sample was harvested and fractionated as described. Data are expressed as counts per minute per ml.

from the anthelmintic activity of AVM, its complete identification was not pursued further. In the purification process described[6], polyenes would be partially separated in each of the early steps such as filtration and solvent partition. Polyene absorbance was not detected in crude avermectins after chromatography on carbon and aluminum.

### Antibiotic Sensitivity

All the yeast and filamentous fungi, shown in Table 1, were resistant to AVM B1a up to a concentration of 400 $\mu$g/ml. The resistance was independent of medium. In contrast, the data in Table 2 show that the filamentous fungi and the *Saccharomyces cerevisiae* strains grown oxidatively were sensitive to oligomycin. The MIC for these organisms was less than 0.75 $\mu$g/ml.

### Effect on Cell Wall Synthesis of *R. miehei*

The incorporation of radioactivity from [1-$^{14}$C]glucosamine into acid-insoluble fraction of growing cells was not inhibited by treating the cells with 1, 10 or 100 $\mu$g/ml of AVM B1a. In contrast, oligomycin at 1 $\mu$g/ml immediately inhibited the incorporation of label (Fig. 1).

Since glucosamine is incorporated into several cell wall fractions, the walls were fractionated to evaluate the drugs specific effect on chitin synthesis. As shown in Table 3, the distribution of isotope in the wall fractions was not altered by treatment with 100 $\mu$g/ml AVM B1a.

### Effect on Cell Well Turnover of *R. miehei*

The radioactivity incorporated during a pulse of [1-$^{14}$C]glucosamine was stable during a chase period of 4 hours in the presence of 100 $\mu$g/ml AVM B1a. Wall fractionation of cells exposed to drug during a 2-hour chase, confirmed that a redistribution of the isotope within the wall had not occurred (Table 4).

## Discussion

Samples of AVM B1a and oligomycin were separated by Sephadex LH-20 and HPLC chromatography. Agar dilution assay of these compounds reconfirmed previous reports that AVM B1a had no detectable antifungal activity up to 400 $\mu$g/ml. Oligomycin inhibited the filamentous fungi and *S. cerevisiae* grown oxidatively at concentrations less than 0.75 $\mu$g/ml. The yeast, *Candida albicans* was resistant to AVM B1a and oligomycin under all conditions. CALCOTT *et al.*[1] reported that AVM inhibited the filamentous fungi but that the yeast, *C. albicans* was resistant. Since the pattern of antifungal activity was comparable to our results obtained with oligomycin, the contradictory results are most likely accounted for by differences in sample preparation and purity.

The results of the experiments described above indicate that AVM B1a does not affect fungal chitin metabolism in *R. miehei*. Incorporation of [1-$^{14}$C]glucosamine into acid insoluble material was not reduced when germinated spores were treated with 100 $\mu$g/ml of AVM B1a for 4 hours. Fractionation of the treated cells confirmed that the drug did not alter the distribution of the isotope in the cell wall polysaccharides during 2 hours when the synthesis and turnover of chitin were measured.

The results presented here show that AVM B1a has no antifungal activity and no affect on fungal chitin synthesis or turnover.

The recent report that AVM affects chitin metabolism in brine shrimp is not inconsistent with the drug's documented effect of reducing the membrane potential. In plants and bacteria, conditions known to reduce the electrical potential are known to affect polysaccharide synthesis[9,10]. It is therefore suggested that chitin metabolism in brine shrimp may be affected as a result of the reduction of membrane potential by AVM treatment.

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