The Mode of Action of AKD-2C, an Antifungal Antibiotic from *Streptomyces* sp. OCU-42815

XU PING, YUKIHIRO AKEDA, KEN-ICHI FUJITA,
TOSHIO TANAKA and MAKOTO TANIGUCHI*

Faculty of Science, Osaka City University,
3-3-138, Sugimoto, Sumiyoshi-Ku, Osaka 558-8585, Japan

(Received for publication April 15, 1998)

The mechanism of the antifungal action of AKD-2C was studied by using *Torulaspora delbrueckii* IFO 1621 as a model. AKD-2C slightly inhibited the incorporation of radioactive precursors into protein, RNA and lipid, but not into DNA. On the other hand, AKD-2C greatly enhanced the leakage of K^+ ions from treated cells and showed a potent effect on liposomal glucose leakage. Using electron microscopic studies, though drastic morphological changes were not observed, an increase in cell membrane irregularities and swelling of the mitochondria caused by AKD-2C were demonstrated. These results suggest that the antifungal action of AKD-2C is due to effects on the yeast cell membrane.

As previously reported[1], *Streptomyces* sp. OCU-42815 produced 4 kinds of monoglycerides AKD-2A, B, C, D. AKD-2A had been reported as an emulsifer[2]; AKD-2B consisting of B_1 and B_2, 2C and 2D were all new monoglycerides. Due to its comparatively good yield, AKD-2C was chosen for studies of its mechanism of antifungal action. In this study, *Torulaspora delbrueckii* IFO 1621 was used as test organism, because of its susceptibility to the inhibitor among all tested fungi.

Materials and Methods

Chemicals

For all experiments, AKD-2C was dissolved in *N*,*N*-dimethylformamide. Radioactive chemicals were purchased from New England Nuclear. Others were of commercial grade.

Antifungal Assay

The antifungal activity of AKD-2C was determined by a two-fold serial broth dilution method[3] after overnight incubation at 25°C using 2.5% malt extract medium.

Cultivation of *T. delbrueckii*

T. delbrueckii was grown with shaking at 25°C in 2.5% malt extract medium, unless otherwise indicated.

Growth Studies

An overnight culture of *T. delbrueckii* IFO 1621 was diluted into fresh medium to give approximately 10^5 CFU/ml. Aliquots of this cell suspension were dispensed into each L-tube which was used to improve the efficiency of shaking culture. After 1-hour incubation, AKD-2C was added and shaking continued. Portions of the culture were withdrawn at intervals to measure the optical density at 660 nm and CFU. CFU were determined by plating dilutions of the culture in saline on malt agar plates and incubating at 25°C for 24 hours.

Macromolecular Synthesis Studies

Radioactive thymine, uracil, leucine or sodium acetate was added to a log-phase culture of *T. delbrueckii* IFO 1621 (10^6 CFU/ml) in glucose medium (0.2% glucose, 0.001% NH_4Cl, pH 6.0) to a final concentration of 2.5 kBq/ml. After 10-minutes incubation with shaking at 25°C, AKD-2C was added and incubation continued. After 1-hour incubation, 400 μl of the suspension was added to 400 μl of ice cold 10% trichloroacetic acid (TCA) and kept on ice for 1 hour. The TCA precipitates were collected on glass-fiber filters (Whatman GF/C) and radioactivity counted in a Beckman liquid scintillation counter with a 5 ml of toluene-based scintillation cocktail consisting of 0.3% 1,4-bis-2-(5-phenyloxazolyl)-benzene and 1% 2,5-diphenyloxazole. The incorporation of radioactive precursors into the acid-insoluble fraction

in control groups is defined as 100%.

To measure the incorporation of acetate into lipid, lipids on the dried filters were further extracted by the procedure described by BLIGH and DYER[4]. A portion of the extracts was transfered to the scintillation cocktail described above and the radioactivity determined.

Cell Permeability Studies

Exponentially growing cells of *T. delbrueckii* IFO 1621 were harvested, washed and suspended in 1/15 M acetic acid buffer (pH 5.2) to give 10^5 CFU/ml. After 10-minutes incubation with shaking at 25°C, AKD-2C was added, incubation continued and at appropriate intervals, portions of the culture were withdrawn and centrifuged. The supernatants were submitted to K^+ ions determinations by flame photometry.

Liposome Studies

The effect of AKD-2C on an artificial membrane was examined by using liposomes prepared from cell suspensions of *T. delbrueckii* IFO 1621, which was heated for 10 minutes in boiling water and then sonicated with glass beads in an ice bath. Lipids were then extracted from the aqueous suspension by the procedure described by BLIGH and DYER[4]. The preparation of glucose-encapsulating liposomes and the assay of trapped glucose leakage from the liposomes were carried out by the methods of KINSKY *et al.*[5].

Electron Microscopic Studies

Both AKD-2C treated and untreated cells (5×10^6 CFU/ml) prepared as described in cell permeability studies were washed with cold saline and fixed in 1% aqueous $KMnO_4$ overnight at 4°C. After centrifugation, the cells were washed twice with distilled water and stained in 1% aqueous uranyl acetate for 1 hour at 4°C. The fixed cells were dehydrated in a series of ethanol concentrations and embedded in Spurr's epoxy resin[6]. Thin sections were cut with a Porter-Blum MT 2B ultramicrotome, stained with lead citrate[7], and examined in a Hitachi H-300 electron microscope.

Results

Inhibitory Activity of AKD-2C against *T. delbrueckii*

We reported previously[1] that AKD-2C showed weak activity against bacteria, yeasts and fungi; however, it showed a relatively strong inhibitory effect on *T. delbrueckii* DSM 70504 (MIC: 12.5 μg/ml). Therefore, we further investigated its antimicrobial activity among 11 strains of *T. delbrueckii*. As shown in Table 1, the activity of AKD-2C was broad. In particular, it was most active against strain IFO 1621 with an MIC of 1.56 μg/ml.

Table 1. Antifungal activity of AKD-2C against *T. delbrueckii*.

Strain	MIC (μg/ml)
DSM 70504	12.5
IFO 0469	3.13
IFO 0704	12.5
IFO 0955	6.25
IFO 1083	6.25
IFO 1129	25
IFO 1172	12.5
IFO 1621	1.56
IFO 1626	3.13
IFO 10245	25
IFO 10573	50

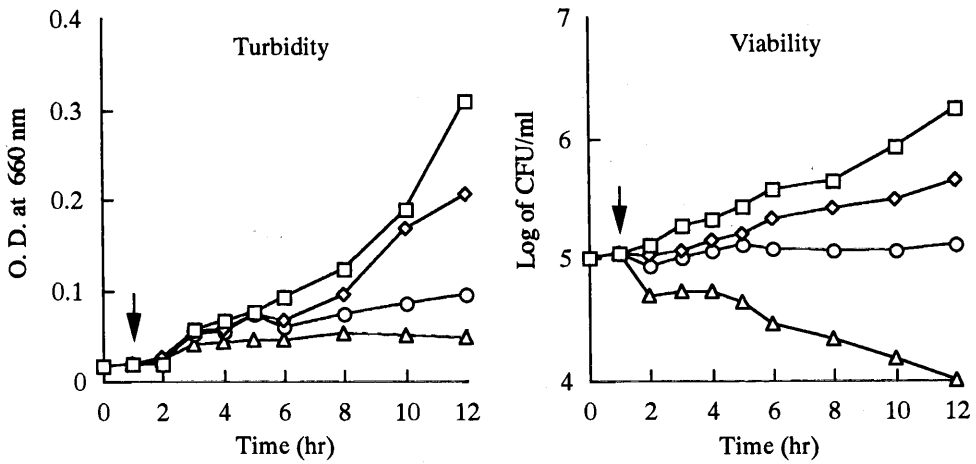
Effect on Growth of *T. delbrueckii*

The growth inhibitory effects of AKD-2C on *T. delbrueckii* IFO 1621 are shown in Fig. 1. When exponentially growing cells were exposed to AKD-2C at 1.56 μg/ml (MIC), increase of turbidity was stopped after 2-hours exposure, while viable cell number did not decrease. At a higher concentration (6.25 μg/ml, 4MIC), there was a reduction of the viable cell number; from 10^5 cell/ml to 10^4 cell/ml after 12-hours incubation. After 24-hours incubation, the viable cell number decreased to 10^3 cell/ml (data not shown). But no reduction in turbidity was observed, indicating no cell-lysis. These results suggested the action of AKD-2C on growth of *T. delbrueckii* seemed to be rather fungicidal than fungistatic without accompanying cell-lysis.

Effect on Synthesis of Major Cellular Constituents

The effect of AKD-2C on DNA, RNA, protein and lipid synthesis in *T. delbrueckii* IFO 1621 cells were examined. As shown in Table 2, after 1-hour exposure at the concentration of 25 μg/ml (equivalent to MIC), the extent of inhibition of incorporation of radioactive precursors employed was maximally 30%, suggesting that the synthesis of major cellular constituents is relatively insensitive to AKD-2C.

Fig. 1. Effect of AKD-2C on growth of *T. delbrueckii* IFO 1621.



The arrows indicate the time of addition of AKD-2C at the following concentrations (μg/ml): □, 0; ◇, 0.39; ○, 1.56; △, 6.25.

Table 2. Effect of AKD-2C on incorporation of radioactive precursors into the acid-insoluble fraction of *T. delbrueckii* IFO 1621 cells.

Radioactive precursors	Percent inhibition of uptake of radioactivity by AKD-2C at 25 μg/ml (equivalent to MIC)
Thymine (DNA)	0
Uracil (RNA)	16.6
Leucine (protein)	29.5
Acetate (lipid)	34.4

K^+ Ions Leakage from *T. delbrueckii* Cells

The effect of AKD-2C on the leakage of K^+ ions from the cells of *T. delbrueckii* is shown in Fig. 2. After 30-minutes exposure to AKD-2C, even at a low concentration of 1.56 μg/ml (MIC), substantial K^+ ions leakage was detected. The amount of leakage seemed to have little relationship to the concentration of AKD-2C.

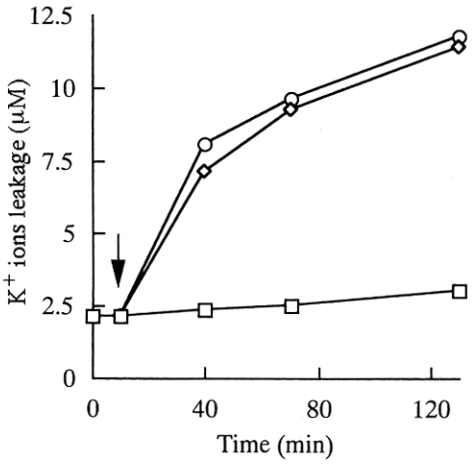
Effect on Liposomes

In order to investigate whether the membrane-active effect of AKD-2C is due to its direct interaction with some components of the yeast cell membrane, the effect of AKD-2C on glucose-encapsulating liposomes prepared with lipids extracted from *T. delbrueckii* IFO 1621 cells was examined. As shown in Fig. 3, after 30-minutes exposure, AKD-2C induced a significant release of trapped glucose from liposomes, at a potency equivalent to amphotericin B, which is known to have selective affinity for sterols[8,9].

Morphological Changes Induced by AKD-2C

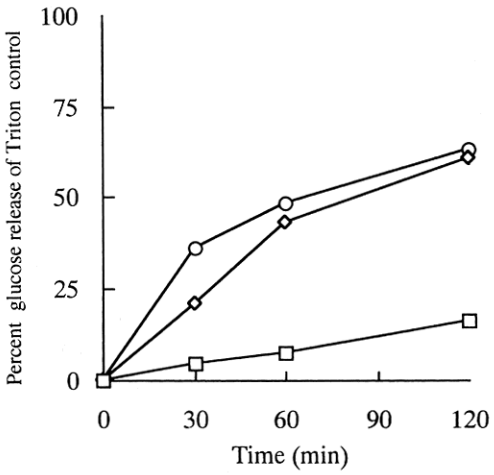
When cell suspensions of *T. delbrueckii* IFO 1621 (5×10^6 CFU/ml) were incubated with 100 μg/ml AKD-2C (nearly equivalent to MIC) for 2 hours, and ultrastructural changes were examined. As shown in Fig. 4, though drastic morphological changes, such as fragmentation of the cell membrane was not observed, increases in irregularity of the cell membrane and swelling of the mitochondria in treated cells were demonstrated, consistent with the results found in cell permeability and liposome studies.

Fig. 2. Effect of AKD-2C on leakage of potassium ions from *T. delbrueckii* IFO 1621 cells.



The arrow indicates the time of addition of AKD-2C at the following concentrations (μg/ml): □, 0; ◇, 1.56; ○, 6.25.

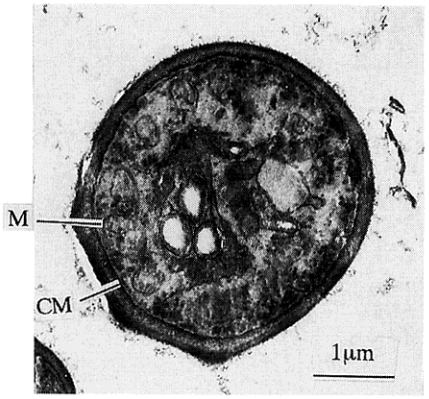
Fig. 3. Effect of AKD-2C and amphotericin B on glucose-encapsulating liposomes prepared from extracted yeast lipids.



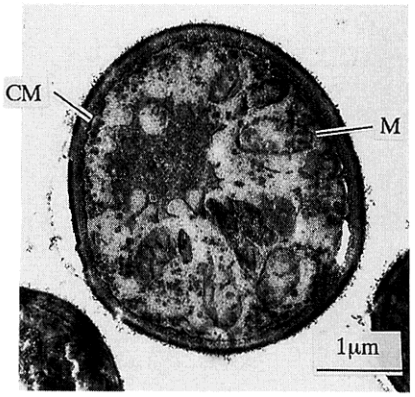
□, 0; ◇, 12.5 μg/ml AKD-2C; ○, 12.5 μg/ml amphotericin B.

Fig. 4. Electron micrographs of untreated and AKD-2C treated *T. delbrueckii* IFO 1621 cells.

CM, cell membrane; M, mitochondrion.



Untreated control cell



Cell treated with 100 μg/ml (nearly equivalent to MIC) of AKD-2C for 2 hours.

Discussion

As shown in above experiments, the effect of AKD-2C on yeast cell growth proved to be fungicidal instead of fungistatic, not accompanying cell-lysis.

In seeking to explain its antifungal action, we first investigated the effects of AKD-2C on macromolecular synthesis. Though AKD-2C inhibited the incorporation of nearly all radioactive precursors to some extent (maximally 30%), This does not explain its antifungal action. Moreover, no inhibitory effect of AKD-2C on endogenous or exogenous cellular respiration was observed (data not shown).

On the other hand, cell permeability studies revealed that AKD-2C caused a rapid loss of K^+ ions from treated cells at low (MIC) and high concentration (4MIC). This leakage seemed not to be dose-dependent.

It has been shown that a representative polyene antifungal antibiotic, amphotericin B having a specific affinity for sterols[8,9] disrupts the cell membrane through its direct interaction with sterols constituting the yeast cell membrane. In a similar manner, AKD-2C induced a significant release of trapped glucose from liposomes prepared from yeast lipids, suggesting that AKD-2C

interacts with the lipid components of the yeast cell membrane. These effects are now under further study.

Electron microscopic studies showed that although drastic morphological changes, such as fragmentation of the cell membrane did not occur, irregularities of the cell membrane and swelling of the mitochondria in treated cells were demonstrated. These morphological changes are consistent with the permeability studies.

Based on the above studies, we propose that the principal action of AKD-2C is on the cell membrane. Detailed studies of its binding site on the cell membrane and other possible effects of AKD-2C are currently in progress.

Acknowledgments

We thank associate Prof. K. IKENISHI for help with electron microscopy and for discussions of the morphological changes.

References

1) AKEDA, Y.; K. SHIBATA, XU PING, T. TANAKA & M. TANIGUCHI: AKD-2A, B, C and D, new antibiotics from *Streptomyces* sp. OCU 42815. Taxonomy, fermentation, isolation, structure elucidation and biological activity. J. Antibiotics 48: 363～368, 1995

2) DUNPHY, P. J. & P. DUNNET (Unilever Research Colworth Lab.): Cosmetic stick. Eur. Pat. Appl. 522 624, Jan. 13, 1993

3) TANIGUCHI, M. & Y. SATOMURA: Structure and physiological activity of carbostyril compounds. Agri. Biol. Chem. 36: 2169～2175, 1972

4) BLIGH, E. G. & W. J. DYER: A rapid method of total lipid extraction and purification. CAN. J. Biochem. Physiol. 37: 911～917, 1959

5) KINSKY, S. C.; J. A. HAXBY, D. A. ZOPF, C. R. ALVING & C. B. KINSKY: Complement-dependent damage to liposomes prepared from pure lipids and Forssman hapten. Biochemistry 8: 4149～4158, 1969

6) SPURR, A. R.: A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruc. Res. 26: 31～43, 1969

7) TANIGUCHI, M.; Y. YANO, E. TADA, K. IKENISHI, S. OI, H. HARAGUCHI, K. HASHIMOTO & I. KUBO: Mode of action of polygodial, an antifungal sesquiterpene dialdehyde. Agric. Biol. Chem. 52: 1409～1414, 1988

8) KRUIJFF, B. D. & R. A. DEMEL: Polyene antibiotic-sterol interaction in membranes of Acholeplasma laidlawii cells and lecithin liposomes. Biochim. Biophys. Acta 339: 57～70, 1974

9) KRUIJFF, B. D.; W. J. GERRITSEN, A. OERLEWANS, R. A. DEMEL & L. L. M. VANDEENEN: Polyene antibiotic sterol interaction in membranes of Acholeplasma laidlawii cells and lecithin liposomes. I. Specificity of the membrane permeability changes induced by the polyene antibiotics. Biochim. Biophys. Acta 339: 30～43, 1974