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UK-2A, B, C and D, Novel Antifungal Antibiotics from *Streptomyces* sp. 517-02

IV. Comparative Studies of UK-2A with Antimycin A_3 on Cytotoxic Activity and Reactive Oxygen Species Generation in LLC-PK1 Cells

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UK-2A, a novel antifungal antibiotic, is a structural relative of antimycin A_3 (AA) and its mode of action is similar to that of AA which inhibits mitochondrial electron transport at complex III. In spite of their structural resemblance, AA had strong cytotoxicity while UK-2A had little cytotoxicity against LLC-PK1 cells as well as other types of cultured cells. When cells were treated with UK-2A or with AA the intracellular ATP content decreased significantly within 5 minutes in glucose-free medium to almost the same extent in both cases. Moreover, under the same conditions, UK-2A killed cells at a similar rate to AA. This suggested that UK-2A entered into the cells and, like AA, inhibited mitochondrial electron transport. On the other hand, AA stimulated reactive oxygen species (ROS) production within 5 minutes even at a low concentration of 1 μM whereas UK-2A did not show such an effect. The difference in the ROS-producing abilities of UK-2A and AA may account for the different cytotoxic effects of the two compounds.

UK-2A was one of the novel antifungal antibiotics isolated from *Streptomyces* sp. 517-02. Its structure is very similar to that of antimycin A_3 (AA).[1~3] Moreover, both compounds inhibit electron transport at complex III in mitochondria from both yeast and rat liver[4]. UK-2A shows very weak cytotoxicity whereas AA is toxic to various kinds of cultured cells, besides being an antibiotic, an insecticide, and a miticide[5].

Our previous report suggested that the low cytotoxicity of UK-2A was due to its limited penetration of the cell membrane[4].

Recent studies have indicated that the cytotoxic activity of AA relates to programmed cell death (PCD) such as apoptosis[6] and secondary necrosis, associated with production of reactive oxygen species (ROS)[7], activation of caspases[8], *de novo* synthesis of ceramide[9] and release of cytochrome *c* into cytosol.[10]

We wish to report here that UK-2A can penetrate the membrane of cultured cells and inhibit mitochondrial electron transport, but there is a difference between UK-2A and AA with respect to ROS production, which is the most rapid event considered as the trigger of PCD in LLC-PK1 cells.

Materials and Methods

Chemicals

Antimycin A_3 and luciferin-luciferase were from Sigma Chemical Co., 2′,7′-dichlorofluorescein diacetate (DCFH-DA) from Acros Organics. Others were commercially available.

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Cell Cultures

Porcine renal proximal tubule cells (LLC-PK1) were obtained from the American Type Culture Collection (CRL 1392; ATCC, Rickville, MD). Cells were regularly grown in 90 mm culture dishes and maintained in a medium composed of Dulbecco's modified Eagle's medium (DMEM; GIBCO Laboratories, Grand Island, NY), 10% heat-inactivated bovine serum and 3.7 g/liter of $NaHCO_3$. Cultures were maintained in a humidified incubator gassed with 5% CO_2-95% air at 37°C and fed at intervals of 24~72 hours. All experiments were performed the day follows cell plating.

Cytotoxic Activities in DMEM

Cytotoxicity of UK-2A and antimycin A were determined using the MTT calorimetric method[11] with minor modification. Briefly, cells were seeded in a 96-well plate and incubated for 24 hours in 100 μl of DMEM containing the appropriate amount of UK-2A or AA. At the end of the incubation period, 20 μl of MTT solution (5 mg/ml in Phosphate Buffered Saline (PBS)) was added and further incubated for 2 hours at 37°C. After removing supernatant, cells were washed with PBS 2 times and the reduced form of MTT was extracted with isopropanol containing 0.1% HCl. The amount of extracted, reduced form of MTT was measured by absorbance at 570 nm using a Shimadzu UV-200 spectrophotometer.

Time Course of Cell Death in Glucose-free DMEM

Cells (1×10^5) were plated in 35 mm culture dishes, washed with glucose-free DMEM and exposed to UK-2A or AA in the same medium for indicated times. At the end of the incubation period, cells were trypsinized and collected by centrifugation.

Cell death was determined using the trypan blue dye exclusion method[12]. Cells failing to exclude the dye were considered to be non-viable. Dead cells were counted with Hepatocytometer (Erma Tokyo 1/10 mm deep). Experiments were performed in triplicate and the values recorded are means ± standard deviations.

Determination of Intracellular ATP Content in Glucose-free DMEM

Cells (5×10^5) were plated in 60 mm culture dish, washed with glucose-free DMEM and incubated with UK-2A or AA for an appropriate time in the same medium. After removing the supernatants, the cells were solubilized by addition of 1.5 ml of 0.14 N HNO_3 at 70°C. The incubation was continued for an additional 20 minutes at 37°C to allow complete solubilization. The supernatants were collected and centrifuged for 10 minutes at $3,000 \times g$. The pH of the supernatant was adjusted to pH 7.0 by adding 0.6 N NaOH in 20 mM Tris[13].

The ATP content was determined using the Luciferin-Luciferase (L/Lase) reaction by counting light emission in a liquid scintillation counter (Beckman LC-6500) on the single photon mode[14]. ATP content is expressed as percent of control. The concentrations of ATP content in control cells were 4.78 nmol/ml/10^5 cells before incubation and 5.90 nmol/ml/10^5 cells after 60 minutes incubation. Experiments were performed three times and the values are means ± standard deviations.

Measurement of Production of ROS in Glucose-free DMEM

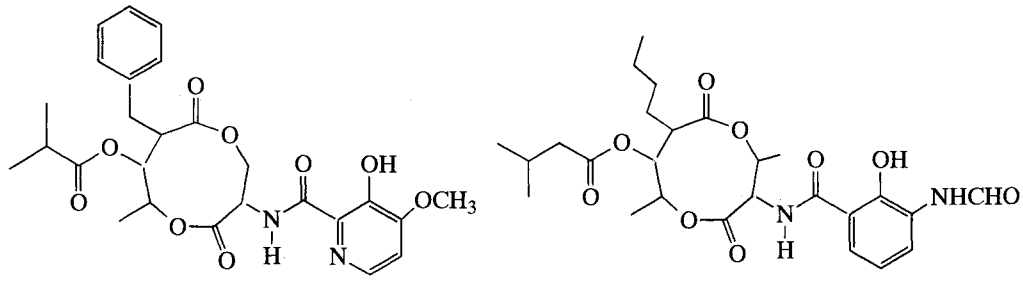
The intracellular generation of ROS was examined using DCFH-DA. This probe measures the generation of ROS[15]. Cells (1×10^5) were introduced to a 24-well plate. After removing the culture medium, the cells were washed with glucose-free modified Gey's buffer (MGB)[16] and incubated with MGB containing 20 μM DCFH-DA for 60 minutes at 37°C. After the incubation period, each well was washed, and 1 ml of MGB was added. Cells were treated with 10 μM UK-2A or AA for different time intervals. At the end of each incubation period, fluorescence intensity of the cell was read using a Cytoflow 2300 (Millipore. Ltd.) with excitation at 488 nm and emission at 525 nm. Experiments were performed three times and the values are means ± standard deviations.

Results and Discussion

As previously described, UK-2A is a structural relative of antimycin A_3 (Fig. 1). The differences in their structures was account for significant differences in biological properties[1]. Table 1 shows cytotoxic effect of UK-2A and AA on several types of cultured cells. UK-2A had little cytotoxicity against LLC-PK1 cells as well as other cells reported previously.[1] AA was cytotoxic even at very low concentrations. This could be attributed to the limited permeability of UK-2A to the cell membrane.

To investigate this problem, intracellular ATP content was measured in glucose-free DMEM. As shown in Fig. 2, it decreased significantly after 5 minutes exposure to 10 μM of UK-2A. After 30 minutes exposure, it reached the same level as for cells treated with AA. Similar effects

Fig. 1. Structures of UK-2A and AA.



UK-2A AA

were observed at lower concentrations by both inhibitors (data not shown). Under glucose-free condition, ATP depletion is widely used as the indicator of the inhibition of electron transport. These cells completely consumed ATP, suggesting that UK-2A can penetrate the cell memtrane and inhibit the mitochondrial electron transport as well as AA does. In DMEM, ATP content in cells treated with both inhibitors remained 80% of the control until 30 minutes. This remaining ATP may be from fermentative production of glucose (data not shown).

In glucose-free DMEM with the addition of 10 μM of UK-2A or AA, cells probably die of ATP deprivation. Many studies suggest that cell death caused by AA relates to the activation of cell death program in addition to less ATP content.[7~11]

Fig. 3 shows the time course of cell death induced by UK-2A and AA in glucose-free DMEM. UK-2A killed cells almost as effectively as AA. After 2 hours exposure to UK-2A and AA, significant injury of the cells was observed under light microscope in contrast to control cells as previously described[17] (data not shown).

Furthermore, the effects of UK-2A and AA on ROS generation in LLC-PK1 cells were examined. Fig. 4A shows that UK-2A did not stimulate ROS generation at an effective concentration of 10 μM (Fig. 2 and 3). By contrast, AA stimulated ROS generation even at 1 μM and the effect was dose dependent. Fig 4. B shows the time course of ROS generation. AA rapidly stimulated ROS generation, whereas UK-2A didn't show such effect.

ROS generation in cells treated with AA is consistent with the previous description[8]. ROS causes lipid peroxidation[18], DNA injury[19], and acts as a signal in PCD[20]. Of the events following the exposure to AA in glucose-free DMEM, ROS generation is the most rapid one[7~10]. Therefore, ROS generated by AA treatment may trigger the cell death program. The cells treated with AA show the necrotic form of cell death. In addition, some apoptotic features are observed in these cells; these include caspase-1 activation, the protective effect on cell death by caspase-3 inhibitor[8] and DNA fragmentation[21]. However, UK-2A did not stimulate ROS generation in LLC-PK1 cells.

Table 1. Cytotoxic activities of UK-2A and AA.

	IC_{50} (μg/ml)	
	UK-2A	AA
p-388	100	0.015
B-16	100	0.02
KB	17	0.063
COLO201	35	0.018
3T3	100	15
LLC-PK1	100	0.10

Complex III of the mitochondrial respiratory chain generates ROS[22], and both UK-2A and AA are complex III inhibitors. The proton motive ubiquinone cycle model (Q-cycle model) of electron flow in the mitochondrial respiratory chain shows that there are two major sites for antibiotic binding in complex III namely the ubiquinone reducing site (Qi) and the ubiquinol oxidizing site (Qo)[23]. Binding of antibiotics to Qi center of the

Fig. 2. Effects of UK-2A and AA on intracellular ATP content in LLC-PK1 cells in glucose-free DMEM.

○ 10 μM UK-2A, ● 10 μM AA.

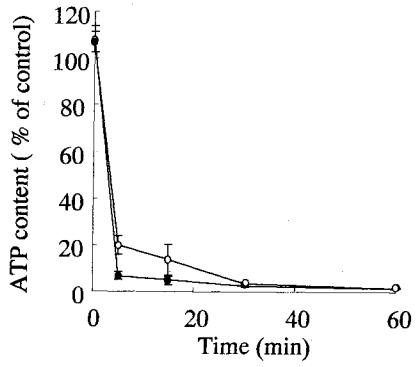


Fig. 3. Time course of LLC-PK1 cell death induced by UK-2A and AA in glucose-free DMEM.

○ 10 μM UK-2A, ● 10 μM AA, ⊙ control.

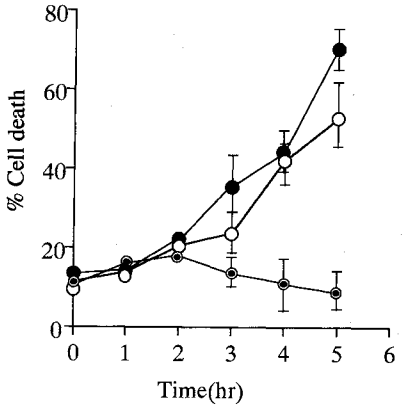
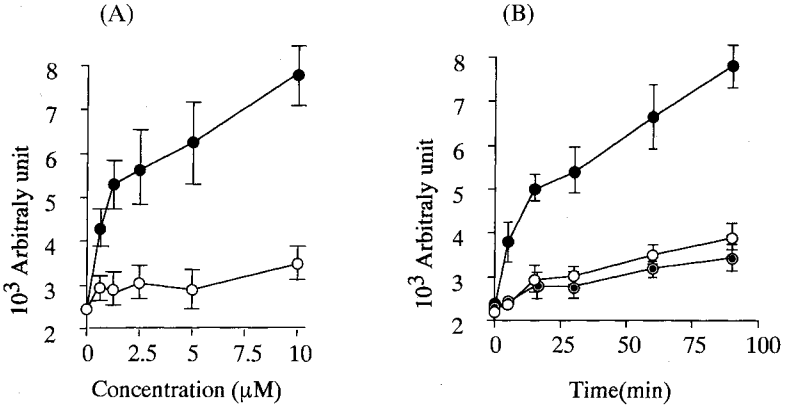


Fig. 4. Effects of UK-2A and AA on ROS generation in LLC-PK1 cells in glucose-free DMEM.

(A) Dose dependency of ROS generation affected by UK-2A and AA. ○ UK-2A, ● AA.
(B) Time course of ROS generation affected by UK-2A and AA. ○ 10 μM UK-2A, ● 10 μM AA, ⊙ control.



complex III causes the accumulation of ubisemiquinone, and to Qo center causes accumulation of ubiquinol. Ubisemiquinone is regarded as a direct electron donor for superoxide formation[22]. AA is a well known Qi center inhibitor[23]. UK-2A penetrated into the cells and inhibited mitochondrial electron transport as shown in Fig. 2 and 3, but it did not stimulate ROS generation. These results suggested that the binding site of UK-2A in mitochondrial complex III is not identical to that of AA. Due to its different blocking way in electron transport chain, UK-2A might not accumulate ubisemiquinone radical, a electron donor to molecular oxygen. Examination of the inhibitory mechanism of UK-2A with isolated complex III is currently underway in our laboratory.

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