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

VIII. Reactive Oxygen Species Generated by C9-UK-2A, a Derivative of UK-2A, in *Rhodotorula mucilaginosa* IFO 0001

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(Received for publication November 1, 2002)

UK-2A was isolated from *Streptomyces* sp. 517-02, which has similarity to antimycin A3 (AA) in structure and inhibitory activities toward electron transport at complex III in mitochondria and has a broad antifungal spectrum1-3). In an attempt to improve the duration of antifungal action of UK-2A, several UK-2A derivatives have been prepared by substituting a nine-membered dilactone ring of UK-2A with an n-alkyl or an isoprenyl moiety4). Previously, we reported a membrane injury caused by a UK-2A derivative, or C9-UK-2A in a strict aerobic yeast *Rhodotorula mucilaginosa* IFO 00015). The patterns of efflux of potassium ions from the yeast cells and of release of enclosed chemicals from artificially prepared liposomes induced by C9-UK-2A were not as rapid as those by amphotericin B5). Therefore, the antifungal activity of C9-UK-2A could not be explained only by a nonspecific membrane injury, whose mechanism is still unclear5).

UK-2A did not stimulate the generation of cellular reactive oxygen species (ROS) in procine renal proximal tubule cells (LLC-PK1) whereas AA did6). This difference depends on the binding manner against dithionite-reduced cytochrome b of complex III7) and might account for their different cytotoxicity6). During our preliminary experiments, AA also stimulated cellular ROS generation in *R. mucilaginosa* IFO 0001 as well as *Saccharomyces cerevisiae* IFO 0203 whereas UK-2A did not.

We tested the effect of C9-UK-2A on cellular ROS generation in *R. mucilaginosa* IFO 0001. C9-UK-2A was prepared and dissolved in *N,N*-dimethylformamide by a previously described method8,9). As shown in Fig. 1, C9-UK-2A generated the ROS nearly proportional to its concentration more than 12.5 μg/ml. Moreover, C9-UK-2A induced the generation time-dependently (data not shown).

Glutathione (GSH)9), one of main antioxidants, is a largest quantity of intracellular SH compounds in budding yeast. To check whether C9-UK-2A gives oxidative stress against *R. mucilaginosa* IFO 0001 cells or not, we investigated GSH/GSSG ratio. In Table 1, GSH/GSSG ratio in the cells treated with C9-UK-2A was about 13% of that in control cells, indicating C9-UK-2A-generated ROS causes oxidation in *R. mucilaginosa* IFO 0001 cells. Its oxidizing activity was three times of that of H2O2.

Lipophilic antioxidant α-tocopherol including α-tocopherolacetate (α-TOH) are naturally occuring ones, which can easily penetrate the plasma membrane and protect free and membranous lipids from oxidative damages, such as lipid peroxidation10). Therefore, we examined the effect of α-TOH on the ROS generation

![Fig. 1. Dose dependent effect of C9-UK-2A on ROS generation in *R. mucilaginosa* IFO 0001 cells in a Sabouraud dextrose (SD) broth.](image-url)

The measurement of cellular ROS generation in *R. mucilaginosa* IFO 0001 was performed by a method dependent on intracellular deacylation and oxidation of 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) to the fluorescent compound 2′,7′-dichlorodihydrofluorescein as described by MACHIDA et al.11). Prior to the measurement of ROS generation, the cells (equivalent to 10^7 cells) were incubated in 1 ml of SD broth11) at 30°C for 30 minutes with 0, 12.5, 25, and 50 μg/ml C9-UK-2A after pretreatment with DCFH-DA for 60 minutes. Values are means±standard deviations (n=3).

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induced by C9-UK-2A in *R. mucilaginosa* IFO 0001 cells (Fig. 2. (a)). α-TOH restricted the C9-UK-2A-generated ROS to 70% at 100 μM and completely suppressed it at 1 mM. Hydrophilic antioxidants, such as ascorbic acid, trolox and N-acetylcystein did not affect the C9-UK-2A-generated ROS (data not shown). It has been reported that fullerene and its derivative generated ROS on photoexcitation and induced significant lipid peroxidation/protein oxidation in membranes and these phenomena could be prevented by antioxidants\(^1\). Therefore, the protective effect of lipophilic antioxidants on C9-UK-2A-generated ROS in *R. mucilaginosa* IFO 0001 cells would suggest the possibility of peroxidation of membranes triggered by C9-UK-2A. On the other hand, a sesquiterpene dialdehyde, polygodial showed a membrane injury accompanying with ROS generation caused by depletion of

<table>
<thead>
<tr>
<th>Addition</th>
<th>Conc.</th>
<th>Total glutathione (nmols /10^7 cells)</th>
<th>GSH (nmols /10^7 cells)</th>
<th>GSSG (nmols /10^7 cells)</th>
<th>GSH/GSSG ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>–</td>
<td>0.17</td>
<td>0.14</td>
<td>0.03</td>
<td>4.7</td>
</tr>
<tr>
<td>C9-UK-2A</td>
<td>25 μg/ml</td>
<td>0.13</td>
<td>0.05</td>
<td>0.08</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>50 μg/ml</td>
<td>0.11</td>
<td>0.04</td>
<td>0.07</td>
<td>0.6</td>
</tr>
<tr>
<td>H2O2</td>
<td>2 mM</td>
<td>0.11</td>
<td>0.07</td>
<td>0.04</td>
<td>1.8</td>
</tr>
</tbody>
</table>

Table 1. Effects of C9-UK-2A and hydrogen peroxide (H2O2) on the total glutathione contents and the ratio of GSH (reduced form) to GSSG (oxidized form) in *R. mucilaginosa* IFO 0001 cells.

**(a) and (b)**: ROS generation was measured as described in Fig. 1. The yeast cells were pretreated with α-TOH for 15 minutes prior to incubation with C9-UK-2A. C9UK indicates incubation with 50 μg/ml C9-UK-2A. Values are means±standard deviations (n=3). (b): The cells were cultivated with 50 μg/ml C9-UK-2A (●), 50 μg/ml C9-UK-2A with 100 μM α-TOH (▲), 50 μg/ml C9-UK-2A with 1 mM α-TOH (▼), 1 mM α-TOH (○), or without drugs (□) in a SD broth with shaking at 30°C. After incubation in SD broth in the presence of the agents indicated, CFU was determined by plating diluted sample on YPD agar plates followed by 48-hour incubation at 25°C before enumerating CFU.
intracellular glutathione\textsuperscript{29}. Polygodial-mediated depletion of glutathione was possibly dependent on a direct interaction between its enal moiety and the sulphydryl group in glutathione by a Michael-type reaction\textsuperscript{31}. As C9-UK-2A does not have any reactive moieties in the molecule, further studies are needed to reveal the correlation between the membrane injury and the ROS generation.

Previously, C9-UK-2A at 100\(\mu\)g/ml was reported to show fungicidal activity after 48-hour treatment\textsuperscript{5}. Here we examined the effect of \(\alpha\)-TOH on decrease in cell viability caused by C9-UK-2A in \textit{R. mucilaginosa} within 8 hours (Fig. 2(b)). \(\alpha\)-TOH at 1 mm almost stopped the decrease in cell viability caused by C9-UK-2A at 50 \(\mu\)g/ml. In \textit{S. cerevisiae} cells, \(\alpha\)-TOH eliminated ROS generated by farnesol\textsuperscript{10} and para-nonylphenol\textsuperscript{13} accompanied by a recovery of the following growth arrest. Although a fungicidal nonyl gallate induced a membrane injury, \(\alpha\)-TOH would not eliminate ROS generated or recover from lethality by its ester\textsuperscript{14}. In this case, the attribution of the membrane injury to the fungicidal effect would be greater than that of ROS generation. \(\alpha\)-TOH could not completely cancel the lethal damage caused by fungicidal drugs, such as nonyl gallate and C9-UK-2A.

The patterns of efflux of potassium ions from yeast cells and of release of enclosed chemicals from artificially prepared liposomes induced by C9-UK-2A were not as rapid as those by typical membrane damaging fungicides, such as amphotericin B\textsuperscript{5}, nonyl gallate\textsuperscript{14} and polygodial\textsuperscript{5}. In addition, the antimicrobial action of C9-UK-2A was limited to eukaryotic cells\textsuperscript{5}. C9-UK-2A gradually decreased the number of CFU of \textit{R. mucilaginosa} IFO 0001 at the concentration of 50 \(\mu\)g/ml (Fig. 2(b)). On the other hand, the typical membrane damaging fungicides did it rapidly. \(\alpha\)-TOH almost stopped the decrease in cell viability caused by C9-UK-2A. These results indicate that antifungal activity of C9-UK-2A does not only depend on a membrane injury. C9-UK-2A stimulated ROS generation dose- and time-dependently. This generation was completely suppressed by 1 mm \(\alpha\)-TOH (Fig. 2(a)). In spite of this fact, cell viability did not recover to control level. Thus, ROS production alone could not fully explain the fungicidal effect of C9-UK-2A. This UK-2A derivative might pull a trigger on lipid peroxidation mediated by ROS generation which might further accelerate the membrane injury.

Acknowledgments

This research was supported by Grant-in-Aids for Scientific Research (B and C) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (12556017 and 14580615), respectively, which are gratefully appreciated. Thanks are also due to the Analytical Division at Osaka City University for the measurements of mass spectra.

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