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Growth Inhibition Dependent on Reactive Oxygen Species Generated by C9-UK-2A, a Derivative of the Antifungal Antibiotic UK-2A, in *Saccharomyces cerevisiae*

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UK-2A is a potent antifungal antibiotic and its structure is highly similar to that of antimycin A_3 (AA). UK-2A and AA inhibit mitochondrial electron transport at complex III. C9-UK-2A, which has been prepared to improve the duration of the antifungal activity of UK-2A, shows durable fungicidal activities against various species of fungi and induces both membrane injury and the generation of cellular reactive oxygen species (ROS) against *Rhodotorula mucilaginosa* IFO 0001 cells. We found that C9-UK-2A inhibited the vegetative growth of *Saccharomyces cerevisiae* IFO 0203 cells accompanying cellular ROS generation in Sabouraud dextrose (SD) medium, which contained a fermentable carbon source. The ROS generation was completely restricted by pretreatment with a lipophilic antioxidant α-tocopherol. In addition, the pretreatment with the antioxidant protected against the growth inhibition induced by C9-UK-2A. C9-UK-2A also induced ROS generation in isolated mitochondria of the *S. cerevisiae* cells. The addition of both a complex I inhibitor rotenone and a complex II inhibitor thenoyltrifluoroacetone reduced ROS generation induced by C9-UK-2A in the whole cells and the isolated mitochondria. The addition of the inhibitors of complex III, AA or myxothiazol, or of complex IV, KCN, did not reduce ROS generation. These results suggest that C9-UK-2A induces ROS generation due to the blockade of electron flow at complex III, thereby inhibiting the growth of *S. cerevisiae* in SD medium.

An antifungal antibiotic, UK-2A, is similar to antimycin A_3 (AA) in both chemical structure and inhibitory activity against the electron transport at complex III in mitochondria[1~4]. The direct binding sites of UK-2A and AA to complex III have been reported to be associated with a 3-hydroxy-4-methoxy-pyridine-2-carboxylyl and a 3-formylamino-salicylyl group, respectively[5,6]. The nine-membered dilactone rings of UK-2A and AA are thought to be needed for their hydrophobicity in order to pass through biomembrane barriers and for the tight binding of the molecules to complex III[5~7]. However, the antifungal effects of UK-2A and AA are temporary because of enzymatic fragility of the dilactone portions[8].

We have replaced the dilactone portion with stable *n*-alkyl and isoprenyl residues in UK-2A derivatives to improve the poor durability of the antifungal activity[8,9]. C9-UK-2A shows the most durable and relatively potent antifungal activity among the derivatives[8]. In this study, we found that C9-UK-2A expressed a growth inhibitory effect on *Saccaromyces cerevisiae* IFO 0203 cells in Sabouraud dextrose (SD) medium, which contains glucose as a respiratory substrate. UK-2A and AA do not express growth inhibitory effects on the yeast cells in such a medium[3]. As *S. cerevisiae* is a facultative anaerobe, the vegetative growth of the yeast is possible through acquisition of energy by alcoholic fermentation even when respiration is blocked. Therefore, as the action of C9-UK-2A on the yeast cannot be explained only by the inhibition of respiration, we investigated in detail the mode of action of C9-UK-2A against *S. cerevisiae* IFO 0203 cells.

Materials and Methods

Strain

A culture *S. cerevisiae* IFO 0203 was purchased from the Institute for Fermentation, Osaka (Osaka, Japan). The cells of this yeast strain were grown in a SD medium (4%

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glucose and 1% Difco Bactopeptone) at 30°C unless stated otherwise.

Chemicals

C9-UK-2A was prepared by a previously described method[9]. Other reagents were of commercial grade. In this study, all antibiotics, including UK-2A derivatives, were dissolved in *N*,*N*′-dimethylformamide prior to biochemical experiments.

Growth Study

The cells of *S. cerevisiae* IFO 0203 were grown overnight in SD medium with vigorous shaking and were diluted into the same medium to give approximately 10^7 cells/ml. Aliquots of this cell suspension were dispensed into each L-tube. After a 15-minute incubation with shaking at 30°C, C9-UK-2A was added to the culture and then the cultivation was continued further. Aliquots of the culture were withdrawn at regular intervals to measure cell turbidity at 610 nm. The cell suspension (10^7 cells/ml) gave a turbidity value of approximately 1.0 at 610 nm.

Cellular Respiration Study

Exponentially growing cells of *S. cerevisiae* IFO 0203 were harvested, washed with 0.9% NaCl and then suspended in 50 mM phosphate buffer (pH 7.0) containing 1% glucose. After 1-hour incubation with shaking at 30°C, the cells were washed and resuspended in the same buffer to give 10^7 cells/ml. At appropriate intervals, aliquots of the suspension were withdrawn. Cellular respiration of the yeast cells in the suspension with or without C9-UK-2A was measured polarographically at 30°C using Hansatech oxygen electrode units[3].

Measurement of ROS Generation

Cellular ROS generation was determined by a method dependent on the intracellular deacetylation and the oxidation of 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) to the fluorescent compound 2′,7′-dichlorodihydrofluorescein (DCF)[10]. After preincubation of the yeast cells (10^7 cells/ml) in SD medium with 40 μM DCFH-DA at 30°C for 60 minutes, aliquots of the cell suspensions (1.0 ml) were withdrawn and further treated with each chemical for the indicated time and then washed and resuspended in 100 μl of phosphate-buffered saline. Fluorescence intensity of DCF in the cell suspension (100 μl) containing 10^7 cells was read with a Cytofluor 2300 fluorescence spectrophotometer (Millipore Co.) with excitation at 480 nm and emission at 530 nm. The arbitrary units was directly based on fluorescence intensity.

Preparation of Yeast Mitochondria

Mitochondria of *S. cerevisiae* IFO 0203 were isolated by the method of GLICK and PON[11]. Protein content was measured by the method of BRADFORD[12] using bovine serum albumin as a standard.

Effect of C9-UK-2A on Yeast Mitochondrial Respiration

The effects of C9-UK-2A on yeast mitochondrial respiration fully stimulated by an uncoupler FCCP were observed by the method of UEKI and TANIGUCHI[3]. Mitochondrial respiration of the yeast in the suspension was measured polarographically at 30°C using Hansatech oxygen electrode units[3].

Measurement of Glutathione

The exponentially growing yeast cells (10^7 cells/ml) derived from an overnight culture were further incubated in SD medium with or without C9-UK-2A at 30°C for 60 minutes, washed, resuspended in ice-cold 8 mM HCl containing 1.3% 5-sulfosalicylic acid, and then broken with glass beads by repeated vortexing. The supernatant obtained after removing the beads and cell debris by centrifugation at 5,000×*g* for 15 minutes at 4°C was used as the cytosolic fraction. Total glutathione content, the sum of reduced (GSH) and oxidized (GSSG) glutathione was estimated as follows. GSSG in the cytosolic fraction was converted to GSH by glutathione reductase and GSH was then quantified the spectrophotometric monitoring of 2-nitro-5-thiobenzoate formation from 5,5-dithiobis(nitrobenzoic acid)[13]. In the cytosolic fraction prepared for the measurement of GSSG, GSH was consumed beforehand by the treatment with 2-vinylpyridine at 30°C for 60 minutes and GSSG was then quantified as described above.

Results

Effect of C9-UK-2A on Growth

The growth inhibitory effect of C9-UK-2A on *S. cerevisiae* IFO 0203 cells is shown in Fig. 1. When exponentially growing cells in SD medium were exposed to C9-UK-2A at 25 or 50 μg/ml, the growth rate measured in terms of turbidity was slightly or significantly reduced, respectively. In addition, for the treatment with concentrations of C9-UK-2A higher than 100 μg/ml, reduction of the number of viable cells was not observed (data not shown). Thus, the action of C9-UK-2A on the yeast cells was not fungicidal but fungistastic.

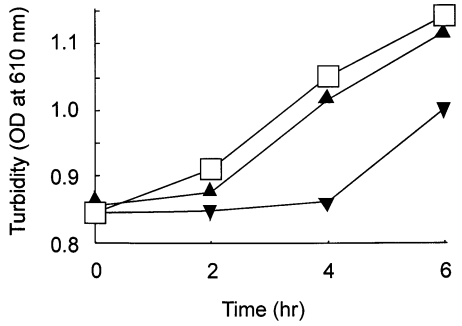
Effect of C9-UK-2A on Cellular Respiration

UK-2A and AA inhibit mitochondrial electron transport at complex III resulting in the inhibition of cellular respiration[3]. Therefore, the effect of C9-UK-2A on cellular respiration of *S. cerevisiae* IFO 0203 was examined. When the yeast cell suspension was incubated with C9-UK-2A, the respiratory activity quickly decreased. Namely, at a concentration of 25 μg/ml, C9-UK-2A caused 92.7% loss of cellular respiration activity after 30 minutes of treatment (data not shown).

Effect of C9-UK-2A on Intracellular Glutathione Content

Glutathione, a primary antioxidant, accounts for the highest quantity of intracellular SH compounds in *S. cerevisiae*[14]. To determine whether C9-UK-2A confers oxidative stress on *S. cerevisiae* IFO 0203 cells, we calculated the GSH/GSSG ratio. In Table 1, the GSH/GSSG ratio in the cells treated with 50 μg/ml C9-UK-2A was 52% of that in control cells, indicating that C9-UK-2A confers oxidative stress on the yeast cells as well as 2 mM H_2O_2. However the decrease in the total glutathione induced by C9-UK-2A was less than that induced by 2 mM H_2O_2. The oxidizing activity of C9-UK-2A was weaker than that of H_2O_2.

Fig. 1. Effect of C9-UK-2A on the growth of *S. cerevisiae*.



Exponentially growing *S. cerevisiae* IFO 0203 cells were grown with shaking in SD broth with 0 (□), 25 (▲), or 50 (▼) μg/ml C9-UK-2A at 30°C.

Effect of C9-UK-2A on Cellular ROS Generation

C9-UK-2A induced ROS generation in a strict aerobic yeast *R. muciraginosa* IFO 0001[15]. We tested the effect of C9-UK-2A on cellular ROS generation in *S. cerevisiae* IFO 0203. As shown in Fig. 2(a), C9-UK-2A induced ROS generation in an almost dose-dependent manner. Moreover, C9-UK-2A induced ROS generation time-dependently (Fig. 2(b)).

Effect of α-TOH on ROS Generation Induced by C9-UK-2A

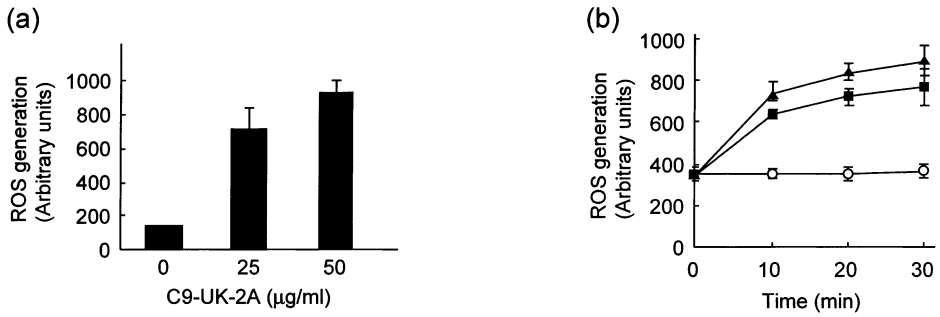
A naturally occurring lipophilic antioxidant α-tocopherol (α-TOH) can easily penetrate plasma membrane and suppress the lipid peroxidation of membranes[16]. We tested the effect of antioxidants on ROS generation induced by C9-UK-2A in *S. cerevisiae* IFO 0203 cells (Fig. 3). One mM α-TOH did not induce ROS generation when added alone. One hundred μM α-TOH suppressed C9-UK-2A-induced ROS generation to control level. On the other hand, a hydrophilic antioxidant, ascorbic acid, which hardly penetrates the plasma membrane of *S. cerevisiae* but acts on the outer surface of the plasma membrane[10,17], did not suppress ROS generation (data not shown). These results suggest that the ROS generation induced by C9-UK-2A does not injure the outer leaflet of the plasma membrane but rather the internal leaflet and/or inside membranes.

Table 1. Effects of C9-UK-2A and hydrogen peroxide on total glutathione content and on the ratio of GSH (reduced form) to GSSG (oxidized form) in *S. cerevisiae* IFO 0203 cells.

Addition	Conc. (μg/ml)	Total glutathione (nmol/10^7 cells)	GSH (nmol/10^7 cells)	GSSG (nmol/10^7 cells)	GSH/GSSG ratio
Control	-	2.26	2.20	0.06	36.7
C9-UK-2A	25	2.08	2.01	0.07	28.7
	50	1.82	1.73	0.09	19.2
Hydrogen peroxide	2 mM	1.16	1.10	0.06	18.3

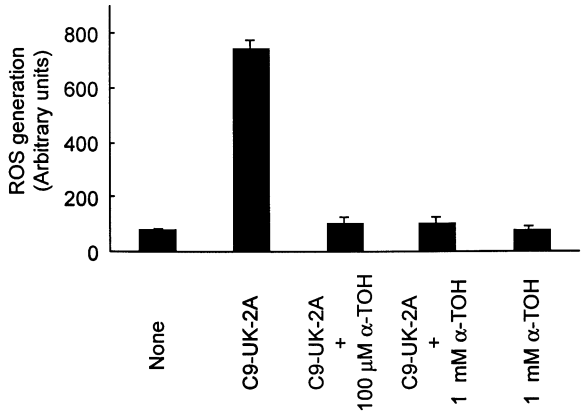
S. cerevisiae IFO 0203 cells (10^7 cells) were grown with or without each chemical at 30°C for 1 hour in SD medium prior to measurement of glutathione content.

Fig. 2. Effects of C9-UK-2A on cellular ROS generation in *S. cerevisiae*.



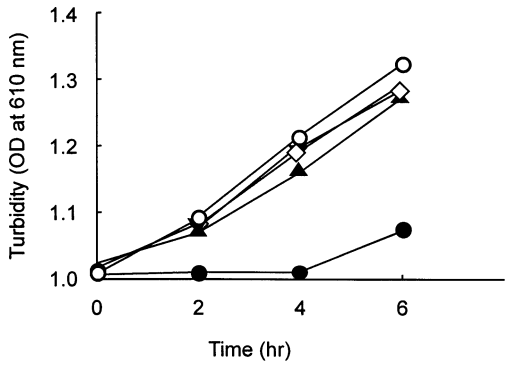
(a) Dose-dependency of ROS generation induced by C9-UK-2A in *S. cerevisiae* IFO 0203 cells. The yeast cells (10^7 cells/ml) were incubated in SD broth with each concentration of C9-UK-2A indicated at 30°C for 30 minutes after pretreatment with DCFH-DA for 60 minutes. ROS generation was measured by using 1 ml of the yeast cell suspension after incubation with C9-UK-2A. (b) Time course of C9-UK-2A-induced ROS generation in *S. cerevisiae* IFO 0203 cells. ROS generation was measured at the indicated times by using 1 ml of the yeast cell suspension (10^7 cells) which had been incubated in SD broth at 30°C with 0(□), 25(■) and 50(▲) μg/ml C9-UK-2A after pretreatment with DCFH-DA for 60 minutes. Values are means ± standard deviations (n=3).

Fig. 3. Effect of α-TOH on ROS generation induced by C9-UK-2A in *S. cerevisiae* IFO 0203.



S. cerevisiae IFO 0203 cells (10^7 cells) were preincubated in SD broth with each concentration of α-TOH indicated for 15 minutes after pretreatment with DCFH-DA for 60 minutes. After preincubation, the yeast cell suspensions were incubated with 50 μg/ml C9-UK-2A for 30 minutes. Values are means ± standard deviations (n=3).

Fig. 4. Effect of α-TOH on the growth inhibition induced by C9-UK-2A in *S. cerevisiae*.



The cell suspensions (10^7 cells/ml) of *S. cerevisiae* IFO 0203 were incubated in SD broth with 0 (○), 50 (●) μg/ml C9-UK-2A, 50 μg/ml C9-UK-2A + 100 μM α-TOH (▲), 50 μg/ml C9-UK-2A + 1 mM α-TOH (▼), or 1 mM α-TOH (◇) at 30 °C.

Protective Effect of α-TOH on the Growth Inhibition Induced by C9-UK-2A

α-TOH suppresses the growth inhibition of *S. cerevisiae* caused by cellular ROS generation when the cells are treated with farnesol or *p*-nonylphenol[10,17]. We tested the effect of α-TOH on the growth inhibition induced by 50 μg/ml C9-UK-2A in *S. cerevisiae* IFO 0203 (Fig. 4). Although 1 mM α-TOH did not affect the growth of the yeast cells when added alone, it almost completely suppressed the growth inhibition at 100 μM. In addition, 1 mM α-TOH did not suppress the inhibition of O_2 consumption caused by 50 μg/ml C9-UK-2A (Table 2). Taking these results in combination with those obtained above, the growth inhibition induced by C9-UK-2A was not due to inhibition of cellular respiration but ROS generation.

Table 2. Effect of α-TOH on inhibition of cellular respiration induced by C9-UK-2A in *S. cerevisiae* IFO 0203 cells.

	Pretreatment with 1 mM α-TOH	O_2 consumption (nmol O_2/min/10^7 cells)	Inhibition (%)
Control	-	12	0
	+	11.7	3
C9-UK-2A	-	0.87	93
	+	0.87	93

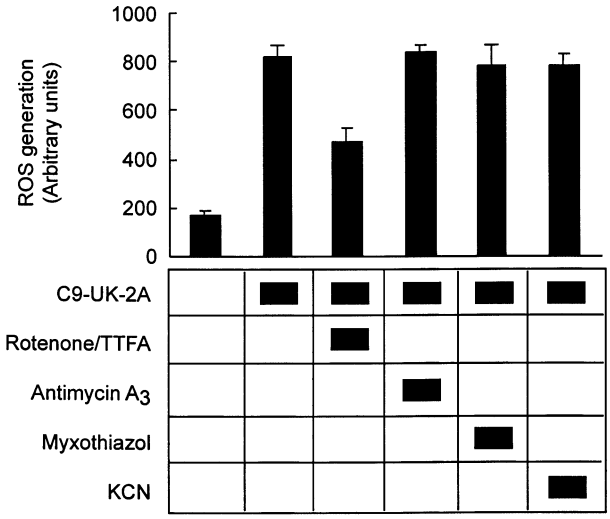
The concentration of C9-UK-2A was 50 μg/ml. Consumption of O_2 was determined after incubation with C9-UK-2A for 60 minutes.

Protective Effect of Blockade in Mitochondrial Electron Transport Chain on C9-UK-2A-induced ROS Generation in Isolated Mitochondria

The majority of cellular ROS are derived from mitochondria[18]. Thus, we confirmed whether C9-UK-2A induced ROS generation in isolated mitochondria of *S. cerevisiae* IFO 0203. C9-UK-2A induced ROS generation at a rate almost proportional at concentrations higher than 25 μg/ml (data not shown).

The protective effect of blockade in the electron transport chain on C9-UK-2A-induced ROS generation in isolated mitochondria of *S. cerevisiae* IFO 0203 is shown in Fig. 5. ROS generation was reduced when the yeast cells were pretreated with both rotenone and TTFA. Pretreatment with AA, MYX, or KCN did not reduce the ROS generation induced by C9-UK-2A in the isolated mitochondria. Rotenone specifically inhibits complex I in the electron transport chain[19], while TTFA is a specific inhibitor of complex II[20]. AA and MYX inhibit the cytochrome reductase of complex III. KCN is a typical inhibitor of complex IV. These results indicate that C9-UK-2A-induced ROS generation is only restricted when electron flow from both complex I and II is limited by rotenone and TTFA.

Fig. 5. Protective effect of blockade in the electron transport chain on C9-UK-2A-induced ROS generation in isolated mitochondria of *S. cerevisiae*.



ROS generation was assayed by using isolated mitochondria of *S. cerevisiae* IFO 0203 with or without the following treatment. Mitochondria were pretreated with 50 μg/ml C9-UK-2A, 50 μg/ml C9-UK-2A + 50 mM rotenone + 1 mM TTFA, 50 μg/ml C9-UK-2A + 20 μM AA, 50 μg/ml C9-UK-2A + 30 μM MYX, or 50 μg/ml C9-UK-2A + 2.5 mM KCN. Bars are means ± standard deviations (n=3).

Discussion

UK-2A and AA strongly restrict the aerobic growth of various filamentous fungi and yeasts by blocking electron transport[3]. Although AA induced cellular ROS generation in porcine renal proximal tubule LLC-PK1 cells thereby expressing cytotoxicity, UK-2A did not[21]. In the yeast *S. cerevisiae*, the tendency of UK-2A and AA to induction of ROS generation seems to be similar to that in mammalian cell lines as described below. C9-UK-2A, which has been prepared to improve the poor duration of the antifungal activity of UK-2A, induces ROS generation and membrane injury promoting the efflux of potassium ions in a strict aerobic yeast *R. muciraginosa* thereby killing the cells[8,15]. Although α-TOH completely eliminated the ROS generation, only partial recovery of the growth was

observed[15]. In *S. cerevisiae* cells, α-TOH eliminated farnesol- and *p*-nonylphenol-induced ROS generation accompaning suppression of growth inhibition[10,17]. Fungicidal Octyl and nonyl gallates also induced membrane injury and ROS generation in *S. cerevisiae*[22,23]. In this case, α-TOH did not eliminate the ROS generation or the lethality of these compounds[22,23]. Therefore, α-TOH could not eliminate the ROS generation induced by fungicidal drugs causing membrane injury.

C9-UK-2A does not inhibit cellular respiration in *R. muciraginosa*[8]. In *S. cerevisiae*, C9-UK-2A exhibited a growth inhibitory effect (Fig. 1) and strictly restricted respiration. In this study, SD medium was used for the cultivation of the yeast strain. As this medium contains glucose as a carbon source, the inhibition of respiration was not expected to affect the growth of such a facultative anaerobic yeast. α-TOH completely restored the growth arrest induced by C9-UK-2A in *S. cerevisiae* (Fig. 4). However this antioxidant could not protect against the inhibition of respiration (Table 2). Interestingly, C9-UK-2A induced ROS generation (Fig. 2) and α-TOH completely eliminated ROS generation (Fig. 3) in *S. cerevisiae*. In addition, the oxidative stress caused by C9-UK-2A against the yeast cells was supported by the decrease in the intracellular GSH/GSSG ratio (Table 1). These results suggest that the growth inhibition caused by C9-UK-2A against *S. cerevisiae* depends on the ROS generation resulting from the inhibition of respiration. On the other hand, the protective effect of α-TOH against C9-UK-2A-induced growth inhibition could be explained by the elimination of the ROS generated as a result of the inhibition of respiration.

C9-UK-2A induced ROS generation in the isolated mitochondria of *S. cerevisiae*. Complex I and complex III of the electron-transport chain are the major sites of cellular ROS generation[24,25]. The inhibition of cellular respiration by AA and MYX is directly involved in the ROS generation at complex III. AA and MYX are typical inhibitors of ubiquinone reducing site (Qi) and ubiquinol oxidizing site (Qo) inhibitors, respectively, at complex III. In fact, when *S. cerevisiae* IFO 0203 cells were treated with 20 μM AA and 30 μM MYX alone, ROS generation was 424±31 and 627±30 arbitrary units, respectively. The ROS generation induced by C9-UK-2A was restricted only by the addition of both TTFA and rotenone. AA, MYX, or KCN did not affect the ROS generation (Fig. 5). In addition, C9-UK-2A inhibited mitochondrial respiration using β-hydroxybutyrate and succinate as a respiratory substrate, but no inhibition was observed using ascorbate-reduced tetramethyl *p*-phenylenediamine as the substrate, indicating that the site of the respiratory inhibition is complex III of mitochondrial electron transport, as is the case for UK-2A (data not shown). Therefore, in the case of C9-UK-2A, the generation site of the ROS was predicted to be complex III.

UK-2A does not induce ROS generation against two yeast strains, *R. mucilaginosa* IFO 0001 and *S. cerevisiae* IFO 0203 (data not shown). However, C9-UK-2A induced ROS generation against these yeasts. This difference between UK-2A and C9-UK-2A may depend on their chemical structures. As previously reported, the nine-membered dilactone rings of UK-2A and AA are not important for binding complex III[5,6]. The replacement of the dilactone ring of UK-2A by a *n*-nonyl residue could change its physiological effects. Therefore the dilactone ring of UK-2A possibly attributes the inhibition of cellular respiration without ROS generation. UK-2A, similar to AA, is an inhibitor of the Qi site[26]. However, the spectral change for dithionite-reduced cytochrome *b* induced by UK-2A binding differed from that induced by AA binding[26]. This difference in binding manner might affect ROS generation at complex III. The replacement of the dilactone ring of UK-2A by a *n*-nonyl residue might generate a UK-2A derivative which functions like an AA-type of Qi inhibitor.

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References

1) Ueki, M.; K. Abe, M. Hanafi, K. Shibata, T. Tanaka & M. Taniguchi: UK-2A, B, C and D, novel antifungal antibiotics from *Streptomyces* sp. 517-02. I. Fermentation, isolation and biological properties. J. Antibiotics 49: 639～643, 1996
2) Hanafi, M.; K. Shibata, M. Ueki & M. Taniguchi: UK-2A, B, C and D, novel antifungal antibiotics from *Streptomyces* sp. 517-02. II. Structural elucidation. J. Antibiotics 49: 1226～1231, 1996
3) Ueki, M. & M. Taniguchi: The mode of action of UK-2A and UK-3A, novel antifungal antibiotics from *Streptomyces* sp. 517-02. J. Antibiotics 50: 1052～1057, 1997
4) Shibata, K.; M. Hanafi, J. Fujii, O. Sakanaka, K. Iinuma, M. Ueki & M. Taniguchi: UK-2A, B, C and D, novel antifungal antibiotics from *Streptomyces* sp. 517-02. III. Absolute configuration of an antifungal antibiotic, UK-2A, and consideration of its conformation. J. Antibiotics 51: 1113～1116, 1998

5) MIYOSHI, H.; N. TOKUTAKE, Y. IMAEDA, T. AKAGI & H. IWAMURA: A model of antimycin A binding based on structure-activity studies of synthetic antimycin A analogues. Biochim. Biophys. Acta 1229: 149～154, 1995
6) UEKI, M.; K. MACHIDA & M. TANIGUCHI: Antifungal inhibitors of mitochondrial respiration: Discovery and prospectives for development. Current Opinion in Antiinfective Investigational Drugs 2: 387～398, 2000
7) USUKI, Y.; K. GOTO, T. KISO, K. TANI, X. PING, K. FUJITA, H. IIO & M. TANIGUCHI: UK-2A, B, C and D, novel antifungal antibiotics from *Streptomyces* sp. 517-02 VI (2). Structure-activity relationships of UK-2A. J. Antibiotics 55: 607～610, 2002
8) TANI, K.; Y. USUKI, K. MOTOBA, K. FUJITA & M. TANIGUCHI: UK-2A, B, C, and D, novel antifungal antibiotics from *Streptomyces* sp. 517-02 VII. Membrane injury induced by C9-UK-2A, a derivative of UK-2A, in *Rhodotorula mucilaginosa* IFO 0001. J. Antibiotics 55: 315～321, 2002
9) USUKI, Y.; K. TANI, K. FUJITA & M. TANIGUCHI: UK-2A, B, C and D, novel antifungal antibiotics from *Streptomyces* sp. 517-02. VI(1). Structure-activity relationships of UK-2A. J. Antibiotics 54: 600～602, 2001
10) MACHIDA, K.; T. TANAKA, K. FUJITA & M. TANIGUCHI: Farnesol-induced generation of reactive oxygen species *via* indirect inhibition of the mitochondrial electron transport chain in the yeast *Saccharomyces cerevisiae*. J. Bacteriol. 180: 4460～4465, 1998
11) GLICK, B. S. & L. A. PON: Isolation of highly purified mitochondria from *Saccharomyces cerevisiae*. Methods Enzymol. 260: 213～223, 1995
12) BRADFORD, M. M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248～254, 1976
13) FLATTERY-O'BRIEN, J. A. & I. W. DAWES: Hydrogen peroxide causes RAD9-dependent cell cycle arrest in G2 in *Saccharomyces cerevisiae* whereas menadione causes G1 arrest independent of RAD9 function. J. Biol. Chem. 273: 8564～8571, 1998
14) ELSKENS, M. T.; C. J. JASPERS & M. J. PENNINCKX: Glutathione as an endogenous sulphur source in the yeast *Saccharomyces cerevisiae*. J. Gen. Microbiol. 137: 637～644, 1991
15) TANI, K.; Y. USUKI, K. FUJITA & M. TANIGUCHI: 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. J. Antibiotics 56: 314～317, 2003
16) MATSURA, T.; K. YAMADA & T. KAWASAKI: Antioxidant role of cellular reduced coenzyme Q homologs and alpha-tocopherol in free radical-induced injury of hepatocytes isolated from rats fed diets with different vitamin E contents. Biochim. Biophys. Acta 1127: 277～283, 1992
17) OKAI, Y.; K. HIGASHI-OKAI, K. MACHIDA, H. NAKAMJURA, K. NAKAYAMA, K. FUJITA, T. TANAKA, S. OTANI & M. TANIGUCHI: Protective effect of antioxidants against para-nonylphenol-induced inhibition of cell growth in *Saccharomyces cerevisiae*. FEMS Microbiol. Lett. 185: 65～70, 2000
18) CHEN, Q.; E. J. VAZQUEZ, S. MOGHADDAS, C. L. HOPPEL & E. J. LESNEFSKY: Production of reactive oxygen species by mitochondria: central role of complex III. J. Biol. Chem. 278: 36027～36031, 2003
19) PALMER, G.; D. J. HORGAN, H. TISDALE, T. P. SINGER & H. BEINERT: Studies on the respiratory chain-linked reduced nicotinamide adenine dinucleotide dehydrogenase. XIV. Location of the sites of inhibition of rotenone, barbiturates, and piericidin by means of electron paramagnetic resonance spectroscopy. J. Biol. Chem. 243: 844～847, 1968
20) INGLEDEW, W. J. & T. OHNISHI: The probable site of action of thenolytrifluoracetone on the respiratory chain. Biochem. J. 164: 617～620 1977
21) TAKIMOTO, H.; K. MACHIDA, M. UEKI, T. TANAKA & M. TANIGUCHI: 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. J. Antibiotics 52: 480～484, 1999
22) FUJITA, K. & I. KUBO: Antifungal activity of octyl gallate. Int. J. Food Microbiol. 79: 193～201, 2002
23) FUJITA, K. & I. KUBO: Plasma membrane injury induced by nonyl gallate in *Saccharomyces cerevisiae*. J. Appl. Microbiol. 92: 1035～1042, 2002
24) TURRENS, J. F. & A. BOVERIS: Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria. Biochem. J. 191: 421～427, 1980
25) SUGIOKA, K.; M. NAKANO, H. TOTSUNE-NAKANO, H. MINAKAMI, S. TERO-KUBOTA & Y. IKEGAMI: Mechanism of O_2-generation in reduction and oxidation cycle of ubiquinones in a model of mitochondrial electron transport systems. Biochim. Biophys. Acta 936: 377～85, 1988
26) MACHIDA, K.; H. TAKIMOTO, H. MIYOSHI & M. TANIGUCHI: UK-2A, B, C and D, novel antifungal antibiotics from *Streptomyces* sp. 517-02. V. Inhibition mechanism of bovine heart mitochondrial cytochrome *bc*1 by the novel antibiotic UK-2A. J. Antibiotics 52: 748～753, 1999