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NOTES

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

VI (3). Role of Substituents on Dilactone Ring of UK-2A and Antimycin A_3 against Generation of Reactive Oxygen Species in Porcine Renal Proximal Tubule LLC-PK_1 Cells

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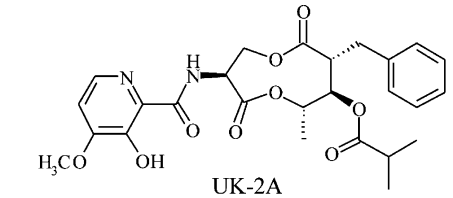
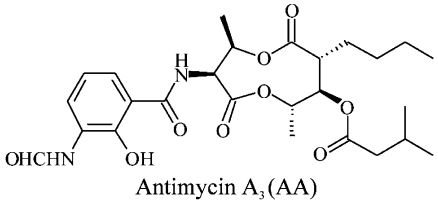
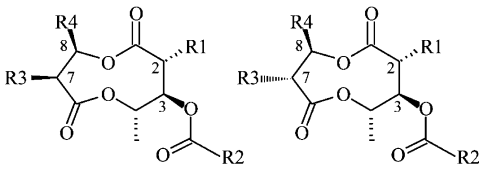
UK-2A, B, C and D (UK-2s) were isolated from *Streptomyces* sp. 517-02[1,2]. They are similar to antimycin A_3 (AA) in chemical structure and inhibitory activity towards electron transport at complex III in mitochondria[3]. However, the benzyl group at the C2 position in UK-2A has never been found in antimycins and a methyl group is lacking at the C8 position (Fig. 1). In addition, UK-2A has a 3-hydroxy-4-methoxypicolinic moiety, while antimycins have a 3-formamidosalicylic moiety which is essential for the inhibition of electron transfer between cytochromes *b* and c_1 in the mitochondrial respiratory chain[4]. Furthermore, UK-2s exhibit potent antifungal activities against human and plant pathogenic filamentous fungi, but they are less cytotoxic than AA against several yeasts including *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Torulaspora delbrueckii* and several mammalian cell lines including mouse leukemia P388, mouse melanoma B16, human oral epidermoid carcinoma KB and human colon adenocarcinoma COLO201. In our studies on UK-2A[5~8], we have been interested in establishing structure-activity relationships among UK-2A analogues. Recently, we have reported the synthesis of UK-2A analogues in which a nine-membered dilactone residue was substituted with several alkyl or isoprenyl moieties, and their biological effects including reactive oxygen species (ROS) generation[9~13]. Here we report the role of the substituents on the dilactone ring of UK-2A and AA against ROS generation in porcine renal proximal tubule LLC-PK_1 cells.

UK-2A-AA hybrids have been prepared previously[11]. Their structures are presented in Fig. 1. The level of cellular ROS generation against LLC-PK_1 cells was measured by a method dependent on the intracellular deacylation and oxidation of 2′,7′-dichlorodihydrofluorescein diacetate to the fluorescent compound 2′,7′-dichlorodihydrofluorescein (DCF)[6]. AA

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







	1	*epi*-1	2	*epi*-2	3	4	5	6	7
R1	A[a]	A	U[b]	U	A	U	A	U	A
R2	U	U	A	A	A	U	U	A	A
R3	U	U	U	U	U	A	A	A	A
R4	U	U	U	U	U	U	U	U	U

a Moiety from UK-2A

b Moiety from AA

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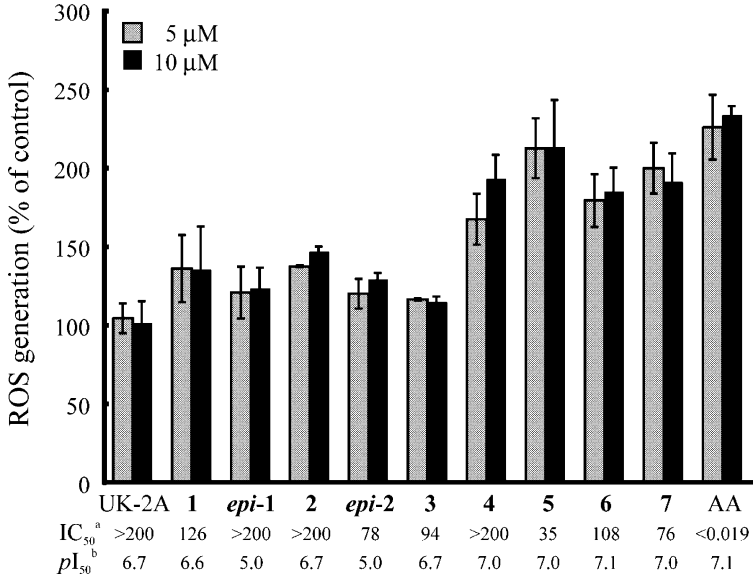
Fig. 2. ROS generation against LLC-PK$_1$ cells treated with UK-2A, AA and their synthetic hybrid derivatives.



Cell cultures were treated with each drug for 1 hour. Cellular ROS generation was measured on the basis of the fluorescence intensity of oxidized DCF. Results were expressed as means±S.D. of three independent observations. Control level is indicated as 100%.

[a] Cytotoxicity (μM), see reference 11.

[b] Respiratory inhibition (the log of the reciprocal I_{50}), see reference 11.

and its derivatives **4**～**7** stimulated ROS generation as shown Fig. 2. They have a 3-formamidosalicylic moiety at the C7 position. The levels of ROS generation induced by AA at 5 and 10 μM were the highest among the derivatives tested and 2.3-fold of the control. On the other hand, UK-2A and its derivatives **1**～**3**, ***epi*-1** and ***epi*-2** did not greatly stimulate ROS generation. They have a 3-hydroxy-4-methoxypicolinic moiety. These results indicate that a 3-formamidosalicylic moiety contributes to ROS generation. In addition, the level of ROS generation among the derivatives correlated with the intensity of respiratory inhibition as shown in Fig. 2. ROS generation was nearly equal to the control level for the derivatives in which the pI_{50} value of respiratory inhibition was less than 6.7. In C7 epimers, ***epi*-1** and ***epi*-2**, a stereochemical mismatch at the C7 position that interferes with their binding to the active site of complex III would be associated with the extremely weak inhibition of respiration, but ROS seemed to be slightly generated. The relation between respiratory inhibition and ROS generation is obscure in this case. AA and the 4 derivatives **4**～**7** showed pI_{50} values more than 7.0 and they significantly induced ROS generation. The substitution at the C2, C3 or C8 position of a nine-membered ring slightly affected ROS generation for 3-formamidosalicylic acid derivatives (**4**～**7**). This suggests that the substituents on the nine-membered dilactone ring moiety themselves do not affect ROS generation or respiratory inhibition in the 3-formamidosalicylic acid derivatives.

The majority of ROS generation is thought to be derived from mitochondria[14]. UK-2A and AA inhibit the electron transport chain at mitochondrial complex III. The mitochondrial generation of ROSs is due to the reduction of O_2 by an electron that leaks from the unstable ubiquinone semiquinone anions formed during redox cycling of ubiquinones present in complex III[15]. Therefore, the ROS generation site is predicted to be complex III judging from the correlation between the level of ROS generation and the intensity of respiratory inhibition. In our previous report, the difference between the ROS-producing abilities of UK-2A and AA may account for the difference between the cytotoxic effects of the two compounds. In this study, the levels of ROS generation induced by **4** at 5 and 10 μM were 1.7- and 1.9-fold that of control, respectively, while the

cytotoxicity of **4** against LLC-PK_1 cells was not observed up to 200 μM. This result indicates that strict respiratory inhibition and the succeeding ROS generation do not directly result in cytotoxicity. In addition, AA showed the highest cytotoxicity among the 3-formamidosalicylic acid derivatives. The methyl group at the C8 position contributes to the potency of the cytotoxic activities among the 3-formamidosalicylic acid derivatives.

The LLC-PK_1 cells treated with the derivatives tested in this study showed morphologies similar to necrotic cell death under microscopic observation (data not shown). However, it has been reported that AA induces the activation of caspases[16] and DNA fragmentation[16], which are typical apoptotic responses. It would partly depend on ROS generation stimulated by respiratory inhibition. Recently, targets of an AA molecule other than complex III have been reported. These targets belong to Bcl-2 family proteins, which are positive and negative regulators of apoptosis progression[17]. They involve one or more conserved Bcl-2-homology domains (BH1-4)[17]. AA is predicted to interact with the BH-3 domain-binding hydrophobic groove of Bcl-2[18]. AA selectively induces apoptosis in cells overexpressing Bcl-2, suggesting that hydrophobic groove-binding compounds including AA may act as selective apoptotic triggers in tumor cells[19]. Therefore the interaction of proteins carrying the BH3 domain, in addition to the blockade of electron transfer at complex III, may be necessary for the cytotoxicity of AA, and such an interaction may be associated with the methyl group at the C8 position.

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