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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

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UK-2A is a potent antifungal antibiotic isolated from *Streptomyces* sp. 517-02 and its structure is highly similar to that of antimycin A. We investigated the inhibition mechanism of bovine heart mitochondrial cytochrome bc_1 complex by the UK-2A using antimycin A and myxothiazol as the reference inhibitors of ubiquinol oxidation (Q_o) and ubiquinone reduction (Q_i) sites, respectively. The inhibitory potency of UK-2A was about 3-fold less than antimycin A. On the basis of the effects of UK-2A on the reduction kinetics of *b* and c_1 hemes, this compound appeared to be an inhibitor of the Q_i site. However, since spectral changes of dithionite-reduced cytochrome *b* induced by UK-2A binding differed from that of antimycin A, the precise binding manner of UK-2A to the enzyme is not identical to that of antimycin A. It could be concluded that antimycin A binding to cytochrome *b* is primarily decided by structural specificity of the salicylic acid moiety.

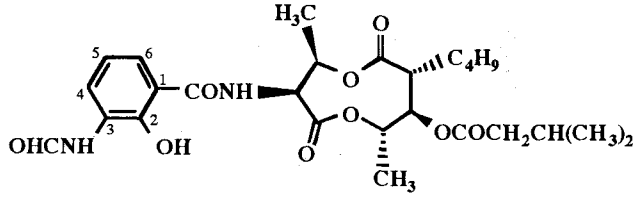
UK-2A was isolated as one of the potent antifungal antibiotics from *Streptomyces* sp. 517-02, and its practical use has been expected in pharmaceutical and agrochemical fields.[1,2] As seen in Fig. 1, the chemical structure of UK-2A is very similar to that of antimycin A (AA), the most potent inhibitor of ubiquinone reduction site (Q_i) of mitochondrial ubiquinol-cytochrome *c* oxidoreductase (cytochrome bc_1 complex). Actually, UK-2A inhibited cytochrome bc_1 complex activity of rat-liver and yeast mitochondria.[3] However, we previously showed that the biological activities of UK-2A differ from those of AA in several respects.[3,4] For instance, although AA is highly toxic to various kinds of mammalian cultured cells, UK-2A elicits very weak cytotoxicity.[1,4] AA, but not UK-2A, stimulates production of reactive oxygen species at cell level.[4] In the present study, to elucidate a reason of the different biological activities between UK-2A and AA, we examined the inhibition mechanism of UK-2A using bovine heart submitochondrial particles (SMP) and isolated cytochrome bc_1 complex.

Materials and Methods

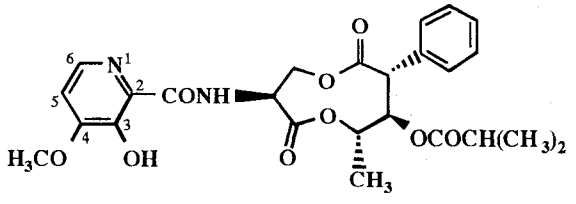
Materials

Antimycin A, myxothiazol and FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrozone) were purchased from

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



Antimycin A



UK-2A

Sigma. SF6847 (3,5-di-*tert*-butyl-4-hydroxybenzylidene malononitrile) was the same sample as that used previously.[5] Other reagents were of the purest grade commercially available.

Preparation of Bovine Heart Submitochondrial Particles

Bovine heart SMP were prepared by the method of MATSUNO-YAGI and HATEFI,[6] and stored at −80°C. The amount of protein concentration of SMP was measured by the method of BRADFORD[7] with bovine serum albumin as the standard.

Preparation of Cytochrome bc_1 Complex

Cytochrome bc_1 complex was isolated according to the method of RIESKE[8] from bovine heart mitochondria and stored at −80°C in the medium consisting of 20% glycerol and 25 mM Tris-HCl buffer (pH 8.0). The concentration of the enzyme was determined by the reduced minus oxidized heme *b* spectrum using 562−575 nm wavelength pair (ε= 28.5 mM^{-1} cm^{-1}).[9]

Assays

The respiration rate of SMP with 10 mM succinate as a respiration substrate was measured with a Clark-type oxygen electrode at 25°C, total volume as 1.0 ml. The reaction medium contained 50 mM potassium phosphate buffer (pH 7.4), 0.25 M sucrose and 1 mM $MgCl_2$. The final protein concentration in the medium was 0.2 mg/ml. The respiratory inhibition was determined from their effects on fully stimulated respiration by 2 μM FCCP. The rate of oxygen consumption without inhibitor was 305 nmol O_2/mg protein per minute.

The redox status of cytochrome *b* in SMP was determined before and after each treatment with the wavelength pair of 563 and 577 nm.[10] The absorbance spectra were measured with a Shimadzu UV3000 spectrophotometer at 25°C. The reaction medium was the same as that used for the respiration experiment except that 1.5 μM SF 6847 was used as an uncoupler. The final protein concentration of SMP was 0.8 mg/ml.

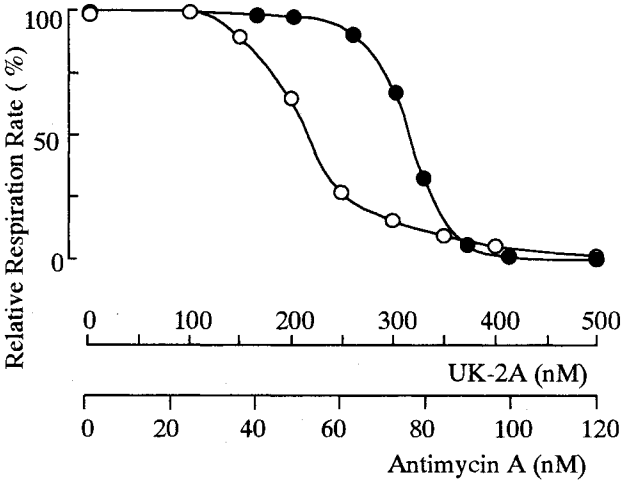
The redox status of cytochrome c_1 in SMP was identified with the wavelength pair of 553 and 539 nm.[10] The reaction medium was the same as that used for the cytochrome *b* experiment except that 1 mM KCN was included. The final protein concentration of SMP was 1.2 mg/ml.

Measurement of the Red-shifts of Reduced Cytochrome bc_1 Complex

Red-shift spectra of dithionite-reduced cytochrome bc_1 complex induced by inhibitor binding were measured with Shimadzu UV2200 spectrophotometer at 25°C as the spectral difference of the dithionite-reduced enzyme with and without a saturated amount of inhibitor. The reaction medium contained 50 mM potassium phosphate (pH 7.4), 0.25 M sucrose, 1 mM $MgCl_2$, 2 mM NaN_3 and 0.05% Triton X-100. The concentration of the enzyme was 8.0 μM.

Fig. 2. Titration curves for the inhibition of succinate oxidase activity in SMP.

○ UK-2A ; ● antimycin A.



The reaction medium contained 50 mM potassium phosphate buffer (pH 7.4), 0.25 M sucrose, 1 mM $MgCl_2$ and 2 μM FCCP. The final protein concentration in the medium was 0.2 mg/ml.

Results

Figure 2 showed the dose-response curve for the inhibition by UK-2A of cytochrome bc_1 complex activity measured as succinate oxidase activity. As a reference, the titration by AA, the most potent inhibitor of Q_i site of cytochrome bc_1 complex, was shown. The dose-response curve of AA was markedly sigmoidal due to pool function of ubiquinone.[11] The I_{50} value, which is the molar concentration to attain 50% inhibition of the control enzyme activity, of AA was 78 nM. UK-2A showed a slightly sigmoidal dose-response curve, and was about 3-fold less active than AA (I_{50}, 230 nM). Thus, UK-2A appeared to be fairly potent inhibitor of cytochrome bc_1 complex.

To elucidate the inhibition site of UK-2A in the enzyme, we examined the effects of UK-2A on the reduction kinetics of *b* hemes (b_H and b_L, the high and low potential hemes

of the bis-heme cytochrome *b*, respectively) using SMP (Fig. 3). AA and myxothiazol (MYX) were used as the typical Q_i and Q_o inhibitors, respectively. Traces A and B show the extent of *b* hemes reduction by succinate when AA and MYX were added alone, respectively. The horizontal bars show the extent of *b* hemes reduction after the addition of dithionite. None of these inhibitors caused complete inhibition by themselves. The extent of *b* hemes reduction was slightly decreased in the presence of MYX compared to that of AA probably because of incomplete reduction of b_L heme.[12] In agreement with the well-known phenomenon,[13~15] when AA and MYX were added together (*i.e.*, under double-kill conditions), reduction of *b* hemes was remarkably inhibited, but not completely (trace C). Trace D shows the effect of UK-2A on *b* hemes reduction. The *b* hemes were rapidly reduced in the presence of UK-2A, and the extent of *b* reduction was identical to that attained by AA. When UK-2A and AA were added together (trace E), the extent of *b* hemes reduction was identical to that attained solely by either of them. The combination of UK-2A and MYX (trace F) caused least inhibition of *b* hemes reduction compared to that of AA and MYX.

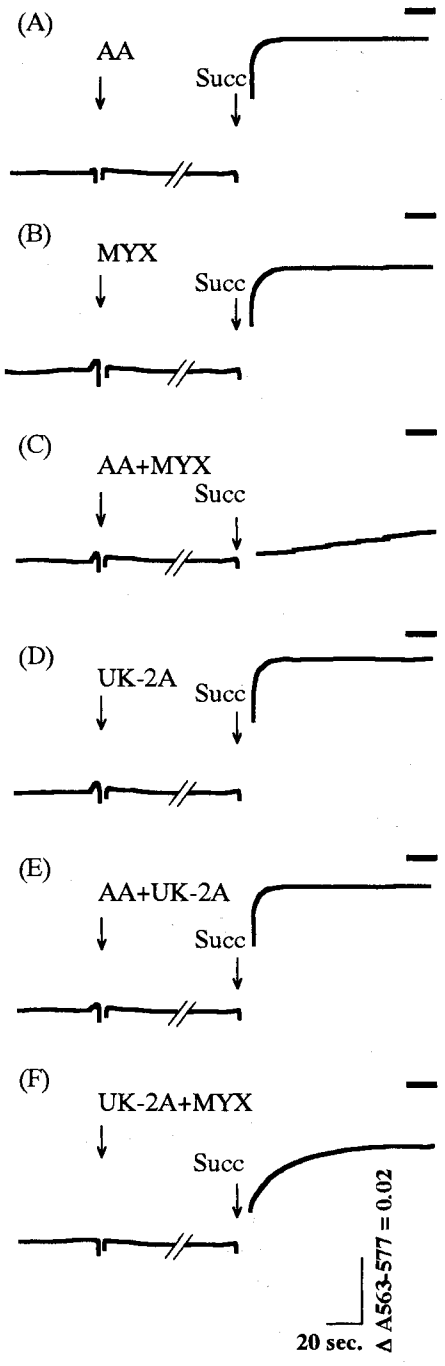
We also examined the effects of the above inhibitor set on the reduction of c_1 heme using SMP (Fig. 4). Traces A and B show the reduction of c_1 heme in the presence of MYX and AA, respectively. MYX almost completely inhibited c_1 heme reduction, whereas rapid c_1 heme reduction was not inhibited by AA.[16] In the presence of UK-2A, reduction of c_1 heme was biphasic, though the final extent of c_1 heme reduction was comparable to that of AA (trace C).

The above observations suggested that the inhibition site of UK-2A is the Q_i site of cytochrome bc_1 complex, although the inhibitory effects on the reduction of *b* and c_1 hemes were not identical to those of AA. We therefore compared the binding manner to isolated cytochrome bc_1 complex between AA and UK-2A (Fig. 5). AA induced red-shift of dithionite-reduced *b* hemes [primarily b_H (b_{562})] by 2~3 nm with a maximum at 564 nm and a minimum at 558 nm, consistent with the previous reports.[10,13] UK-2A also induced the red-shift, whereas a different spectra showed a maximum at 565 nm and a minimum at 561 nm, indicating that the binding manner of UK-2A to cytochrome bc_1 complex differs from that of AA.

Discussion

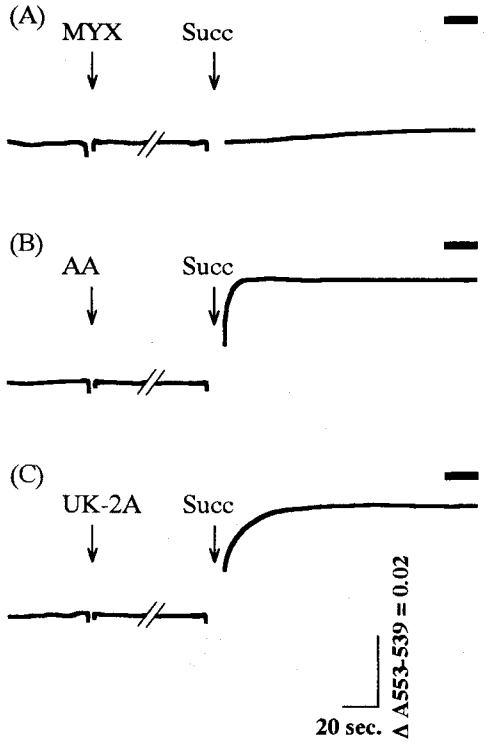
The present results showed that UK-2A is a potent inhibitor of the Q_i site of mitochondrial cytochrome bc_1 complex, whereas its binding manner to the enzyme is not iden-

Fig. 3. Reduction of the *b* hemes by succinate in SMP.



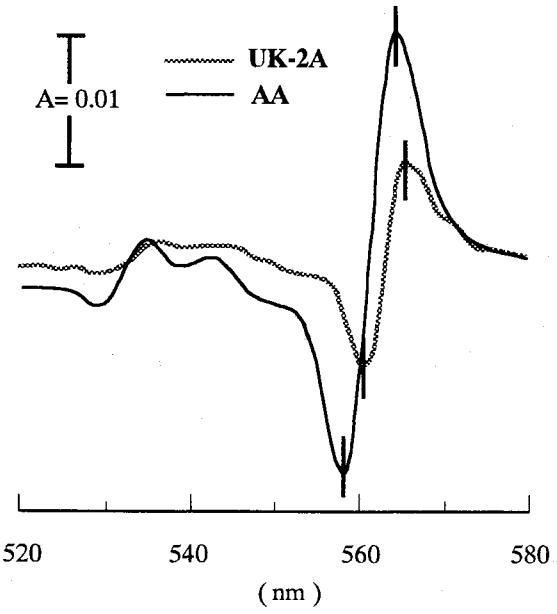
The reduction of *b* hemes was monitored at 563 minus 575 nm. The reaction medium contained 50 mM potassium phosphate (pH 7.4), 0.25 M sucrose, 1 mM $MgCl_2$ and 1.5 μM SF6847. The final protein concentration in the medium was 0.8 mg/ml. Where indicate, 10 mM succinate (Succ), 4 μM antimycin A (AA), 6 μM myxothiazol (MYX) and 10 μM UK-2A were added. The horizontal bar at the right of each trace indicates full reduction of the *b* hemes achieved after addition of a few grains of sodium dithionite ($Na_2S_2O_4$).

Fig. 4. Reduction of the c_1 heme by succinate in SMP.



Effects of antimycin A, UK-2A and myxothiazol on the reduction of cytochrome c_1 by succinate. The reduction of c_1 was monitored at 553 minus 539 nm. Experimental conditions were the same as those described in the legend to Fig. 3, except that 1 mM KCN was included in the reaction medium and the final protein concentration was 1.2 mg/ml. Where indicate, 15 mM succinate (Succ), 6 μM Antimycin A (AA), 9 μM myxothiazol (MYX) and 15 μM UK-2A were added.

Fig. 5. The red-shift spectra of dithionite-reduced isolated cytochrome bc_1 complex induced by inhibitor binding.



The reaction medium contained 50 mM potassium phosphate (pH 7.4), 0.25 M sucrose, 1 mM $MgCl_2$, 2 mM NaN_3 and 0.05% Triton X-100, and the final concentration of the enzyme was 8.0 μM. Inhibitor concentrations: AA, 20 μM; UK-2A, 50 μM.

tical to that of AA. In several aspects, inhibitory effects of UK-2A on the reduction of *b* and c_1 hemes seem not to be thoroughly placed under the category of "Q_i inhibitor" which was historically defined for AA. For instance, the inhibition of *b* hemes reduction in combination of UK-2A and MYX was much weaker than that of AA and MYX (*cf.* traces C and F in Fig. 3), and UK-2A inhibited the reduction of c_1 heme, though the extent of inhibition was much weaker than that of MYX (*cf.* traces A and C in Fig. 4). Nevertheless, we classified UK-2A as a Q_i site inhibitor from the reasons discussed below.

Recently, MATSUNO-YAGI and HATEFI[12,17] closely examined the effects of several cytochrome bc_1 complex inhibitors including AA on the reduction and reoxidation kinetics of *b* and c_1 hemes using SMP. One of the important conclusions driven from the studies is that although AA and MYX elicit maximal inhibition at 1 mol of inhibitor/mol of cytochrome bc_1 complex monomer, they cause inhibition by altering the conformation of the cytochrome *b*, not by blocking a single specific electron transfer step. This proposed inhibitor-induced conformational change of cytochrome *b* as the basis of the pleiotropic inhibitory effect of stoichiometric inhibitors seems to consist with the observations from other laboratories.[18,19],*1 If this is the case, the conformational change induced by inhibitor binding would vary depending upon the inhibitor structure even if they bind to the same domain (Q_i or Q_o site),*2 and conse-

*1 It should be noted that X-ray crystallographic study[21] and resonance Raman spectroscopic study of individual hemes[22] indicated that antimycin A does not induce structural changes in cytochrome *b* molecule except for local changes near the inhibitor binding site.

*2 In this context, the crystal structure of bovine cytochrome bc_1 complex demonstrated that Q_o inhibitors occupy different subsites in the Q_o reaction domain[23].

quently the inhibitory effect on electron transfer in the enzyme would also vary. Actually, in contrast to AA, another Q_i inhibitor HQNO does not completely inhibit *b* hemes reduction even in combination with MYX (*i.e.*, under double-kill conditions).[17] In addition, the extent of inhibition of *b* hemes reduction under double-kill conditions using AA as a Q_i inhibitor significantly differs depending upon Q_o inhibitor used (*e.g.* MYX or MOA-stilbene).[17] Thus, the inhibition behavior of UK-2A would be understood on the same lines. The previous observation that AA, but not UK-2A, stimulates production of intracellular reactive oxygen species[4] may be related to the different inhibitory effects on cytochrome bc_1 complex activity between the two inhibitors.

On the basis of structure/activity relationship of a series of synthetic AA analogues, MIYOSHI and colleagues[16,20] indicated that both a hydroxy and a formylamino groups located in the 2- and 3-positions, respectively, on the salicylic acid moiety of AA are essential structural factors to elicit natural antimycin-like inhibitory action, although the analogues which lack the 3-formylamino group elicit *apparent* inhibition of cytochrome bc_1 complex activity. They also indicated that the dilactone ring moiety is not essential for the inhibition and can be replaced by other hydrophobic structure like substituted diphenylether. Considering these structural requirements, irrespective of markedly similar chemical structures, the precise binding manner of UK-2A should be different form that of AA due to the lack of the 3-formylamino group, as was actually the case. It could be therefore concluded that AA binding to cytochrome *b* is primarily decided by structural specificity of the salicylic acid moiety.

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