The Effect of Nalidixic Acid Group Compounds on Reduction of Cytochrome *c* from Horse Heart and *Candida krusei*

By SHIGERU YAMABE

Research Institute, Kobe College, Nishinomiya-Shi, Hyogo-Ken, Japan

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Reduction of cytochrome *c* from both horse heart and *Candida krusei* by FeSO_4 has been demonstrated. This reaction was stimulated by nalidixic acid and structurally related compounds, and the effect was more pronounced for the yeast cytochrome. Divalent metal ions other than Fe^{2+} lessened or abolished the stimulation by these compounds. Fe^{2+} and other metal ions altered the spectra of nalidixic acid and related compounds indicating the formation of metal chelate complexes. 1,10-Phenanthroline inhibited reduction of cytochrome *c* by Fe^{2+} . Other divalent metal ions relieved the inhibition, probably by forming chelates with 1,10-phenanthroline. These results suggest that metal ion chelation may be involved in the molecular mode of action of nalidixic acid and related drugs. The relevance of this artificial electron transfer system to bacterial electron transfer *in vivo* is discussed.

INTRODUCTION

Nalidixic acid (NA) is a clinically important synthetic antibacterial drug of pyridopyridine structure (Leshner *et al.*, 1962) and is more active against Gram-negative than Gram-positive organisms. It selectively inhibits DNA synthesis in bacteria (Goss, Deitz & Cook, 1964, 1965). Piromidic acid (PA) is a recently developed analogous drug of pyridopyrimidine structure (Minami, Shono & Matsumoto, 1971) and is active against both Gram-negative and Gram-positive organisms (Shimizu, Nakamura & Takase, 1971). In the course of further development of NA group compounds, the electron transfer system from FeSO_4 to cytochrome *c* (Yamabe's system) has proved useful in predicting their antibacterial activity against Gram-positive bacteria. There is an excellent correlation between antibacterial activity and ability to stimulate Yamabe's system (Yamabe, 1976). Unlike NA group compounds, 1,10-phenanthroline (OP) retarded the system markedly and antagonism of OP against NA and PA has been demonstrated in *E. coli* (Yamabe & Shimizu, 1977), using the filter paper strip agar diffusion method (Dye, 1956).

These findings indicated that Yamabe's system might be related to a bacterial electron transport process of importance *in vivo*. However, since cytochrome *c* preparations hitherto used as electron acceptors were purified from horse heart, it seemed desirable to obtain data with a microbial cytochrome *c*.

This paper describes such experiments, the results of which indicate that *Candida* cytochrome *c* is a better electron acceptor in Yamabe's system and that metal ions other than Fe^{2+} are inhibitory. The effects of exogenous Fe^{2+} on the antibacterial activity of NA group compounds are also described.

METHODS

Chemicals. Cytochrome *c* from horse heart (Sigma, type III) and twice recrystallized cytochrome *c* from *Candida krusei* (Sankyo, Tokyo, Japan) were used. NA was obtained from commercial sources. Oxolinic acid (OXA) and AT-1105 were synthesized according to the methods of Kaminsky & Meltzer (1968) and Minami *et al.* (1976), respectively. Pipemidic acid (PPA; Shimizu *et al.*, 1975), AT-1491 and PA were provided by Dainippon Pharmaceutical Co., Osaka, Japan. The structural formulae of these compounds are shown in Fig. 1.

Cytochrome *c* reduction. Non-enzymic reduction of cytochrome *c* by FeSO_4 was measured as the increase in the absorbance at 550 nm under aerobic conditions at 23 °C as described previously (Yamabe, 1976). Reaction mixtures contained: cytochrome *c* (20 μM), FeSO_4 (20 μM) and a NA group compound (80 μM) or OP (100 μM) in 10 mM-potassium/sodium phosphate buffer, pH 7.0. Reaction mixtures to measure the effects of metal ions contained in addition: CoSO_4 , CuSO_4 , NiSO_4 , ZnSO_4 or MnSO_4 (200 μM , except 40 μM for CuSO_4 in the FeSO_4 /PA/cytochrome *c* system).

Spectral measurements. Interactions of Fe^{2+} and other metal ions with NA group compounds were measured by determination of absolute and difference spectra. Cuvettes contained the NA group compound (5 μM) and FeSO_4 (25 μM) or CoSO_4 in phosphate buffer, and measurements were carried out before and after warming for 20 min at 45 °C. For difference spectra, both reference and sample cuvettes contained the NA group compound (5 μM).

All spectroscopic measurements were made with a Shimadzu double beam UV-VIS recording spectrophotometer.

Antibacterial test. The minimum inhibitory concentrations (m.i.c.) of NA, PA and OP in the absence and presence of FeSO_4 were determined by the broth dilution method with *E. coli* NIHJ C-2 as test organism. Various concentrations of drug solution or FeSO_4 -containing drug solution (0.5 ml) were pipetted into aliquots (4.5 ml) of the medium (nutrient broth, pH 7.0) in tubes. They were inoculated with one drop of an overnight culture diluted to give approx. 10^3 viable bacteria per drop, and incubated at 37 °C for 48 h. The m.i.c. was defined as the lowest concentration of drug with which no visible growth was detected.

RESULTS

The effects of NA group compounds on the reduction of *Candida* cytochrome *c* by FeSO_4 are shown in Fig. 2(a). Curve a is a standard time course with no added NA group compound. Curves b to g show the stimulatory activity of NA group compounds. Similar effects were obtained using horse heart cytochrome *c* (Fig. 2b). The relative stimulatory activity of the NA group compounds was similar with both cytochromes but the absolute level of stimulation was somewhat greater with the *Candida* cytochrome.

Figure 3 shows the effects of Co^{2+} and other metal ions on the rate of reduction of *Candida* cytochrome *c* which has been accelerated by PA. Co^{2+} inhibited the reaction, the effect being concentration dependent (not shown in the figure). This indicates competition of Co^{2+} with Fe^{2+} . CuSO_4 (40 μM), NiSO_4 , ZnSO_4 and MnSO_4 (200 μM) also retarded the rate which had been stimulated by PA and this inhibitory activity was in the order:

$$\text{Cu}^{2+} > \text{Ni}^{2+} > \text{Co}^{2+} > \text{Zn}^{2+} > \text{Mn}^{2+}.$$

Ultraviolet (u.v.) absorption spectra of NA group compounds were affected by these metal ions in the absence of cytochrome *c*. For example, one of the absorption peaks (λ_{max} , 275 nm) of PA was markedly increased by warming in the presence of FeSO_4 . Similar spectral changes of PA were obtained in the presence of CoSO_4 or other metal sulphates. These changes were more pronounced in the difference absorption spectra.

Cobalt and other metal ions affected the time course of *Candida* cytochrome *c* reduction which had been retarded by OP (Fig. 4). Curves a and b are standard and reference time courses, respectively. Curves c to i were obtained in the presence of metal ions. The retardation of cytochrome *c* reduction by OP (curves a and b) was inhibited by these metal ions (curves e and g). At the same time, in the case of a weak inhibitor (curves f and h) or a potent inhibitor at low concentrations (curves c and d), the shape of the absorption-time curve was altered. This might be due to a shift in the equilibrium of the reaction, as the final level of

Fig. 1. Structural formulae of NA group compounds.

Fig. 2. Stimulatory effects of NA group compounds ($80 \mu\text{M}$) on *Candida* (a) and horse heart (b) cytochrome *c* reduction by FeSO_4 ($20 \mu\text{M}$). The assay procedure is described in Methods. a, System with no added drug; b to g, systems containing NA, PA, PPA, OXA, AT-1105 and AT-1491, respectively.

absorbance was dependent on the concentration of these potent inhibitors (see Discussion). Thus the inhibitory activity was in the order: $\text{Co}^{2+} \geq \text{Ni}^{2+} > \text{Zn}^{2+} > \text{Cu}^{2+} > \text{Mn}^{2+}$.

FeSO_4 at concentrations above 1.44 mM enhanced the antibacterial activities of NA and PA. On the other hand, FeSO_4 decreased that of OP and the effect was concentration-dependent over a wide range (Table 1).

DISCUSSION

Two main points concerning the mechanism of action of NA group compounds on Yamabe's system arise from this study. One is that a cytochrome *c* of microbial origin (*Candida krusei*) is a better electron acceptor than one of animal origin in the presence of NA group compounds. The other concerns the competitive effects of other metal ions with

Fig. 3

Fig. 4

Fig. 3. Inhibitory effects of metal ions on the PA-stimulated reduction of *Candida* cytochrome *c* by FeSO_4 . a, Standard system without PA and metal ion; b, reference system containing PA but no metal ion; c to g, systems containing CoSO_4 , CuSO_4 , NiSO_4 , ZnSO_4 and MnSO_4 , respectively.

Fig. 4. Inhibitory effects of metal ions on the OP-retarded reduction of *Candida* cytochrome *c* by FeSO_4 . a, Standard system without OP and metal ion; b, reference system containing OP ($100\ \mu\text{M}$) but no metal ion; c to i, systems containing CoSO_4 ($20\ \mu\text{M}$), CoSO_4 ($40\ \mu\text{M}$), and CoSO_4 , CuSO_4 , NiSO_4 , ZnSO_4 , MnSO_4 (all at $400\ \mu\text{M}$), respectively.

Table 1. Effect of FeSO_4 on the m.i.c. values of NA, PA and OP for *E. coli* NIHJ JC-2

M.i.c. values are given in terms of μM or, in parentheses, $\mu\text{g ml}^{-1}$.

Drug	M.i.c. of drug with FeSO_4 at (μM):					
	2880	1440	360	90	22.5	0
NA	6.9 (1.6)	6.9	13.4 (3.1)	13.4	13.4	13.4
PA	10.8 (3.1)	21.7 (6.25)	43.4 (12.5)	43.4	43.4	43.4
OP	8000 (1600)	4000 (800)	1000 (200)	500 (100)	250 (50)	125 (25)

Fe^{2+} when used with NA group compounds as stimulators or OP as a retarder of Yamabe's system.

The order of stimulatory activity of NA group compounds was the same for both cytochromes and, in addition, paralleled those for *in vitro* antibacterial activity against Gram-positive bacteria as reported in the previous study (Yamabe & Shimizu, 1977).

In the absence of cytochrome *c*, Cu^{2+} , Ni^{2+} and Co^{2+} as well as Fe^{2+} caused changes in the spectra of NA group compounds only on warming. These findings suggest that cytochrome *c* facilitates complex formation at room temperature. Though the binding affinities of Cu^{2+} , Ni^{2+} and Co^{2+} with NA group compounds were higher than that of Fe^{2+} , only Fe^{2+} could transfer one electron to cytochrome *c* from its bound state. If Fe^{2+} and another metal ion (M^{2+}) have stability constants K_{Fe} and K_{M} for a common chelator (X), the ratio of the equilibrium concentrations of complexes $\text{Fe}^{2+}\text{-X}$ and $\text{M}^{2+}\text{-X}$ should decrease with increase in $K_{\text{M}}/K_{\text{Fe}}$ when Fe^{2+} and M^{2+} are both present. Thus, when $\text{X} = \text{PA}$, the rate of cytochrome *c* reduction should also decrease, since it is dependent on the concentration of the $\text{Fe}^{2+}\text{-PA}$ complex. However, when $\text{X} = \text{OP}$, the rate should increase with increase in $K_{\text{M}}/K_{\text{Fe}}$, since the equilibrium concentration of the $\text{Fe}^{2+}\text{-OP}$ complex unable to reduce cytochrome *c* decreases.

When EDTA, oxalic acid or 8-hydroxyquinoline are used as chelators, the stability constants of complexes with metal ions are in the order:

(Mellor & Maley, 1948; Irving & Williams, 1948). However, a different order of stability constants was obtained for OP (Sillén & Martell, 1964):

It seems likely that the antibacterial activity of NA group compounds and OP may involve a chelation mechanism. In addition to the u.v. spectral changes, there are close similarities between the orders of inhibitory effects of five metal ions on Yamabe's system containing PA and OP as a stimulator and a retarder respectively, and the orders of stability constants mentioned above.

The effect of Fe^{2+} on the antibacterial activity of 8-hydroxyquinoline (HQ) involved a chelation mechanism but it disappeared when the molar ratio of Fe^{2+} to HQ was 1:3 (Albert, 1958). The effect of Fe^{2+} on spiramycin was due to reductive inactivation which was also observed with cysteine (Banič, 1975). Therefore, these effects of Fe^{2+} on HQ and spiramycin might be not the same in nature as those on NA and OP, respectively.

The effect of Fe^{2+} on the antibacterial activities of NA, PA and OP may reflect interaction with an *in vivo* electron transport system involving reduction of cytochrome *c* by free or bound Fe^{2+} . There is some evidence that the antimicrobial activity of NA may be due partly to an effect on respiratory systems (Dunkle, Van Etten & Bramble, 1972; Carnevali, Sarcoe & Whittaker, 1976). Alternatively, it is possible that the phenomena are due to the effects of chelation on uptake of these antimicrobial agents or some other physiological system requiring Fe^{2+} .

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