ROLE OF THE NAPHTHOQUINONE MOIETY IN THE BIOLOGICAL ACTIVITIES OF SAKYOMICIN A

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The naphthoquinone moiety was proven to be essential to the biological activities of sakyomicin A using various naphthoquinone derivatives. Among the naphthoquinones tested, juglone (5-hydroxy-1,4-naphthoquinone) which resembles the partial structure of sakyomicin A was the most active in cytotoxicity against murine lymphosarcoma L5178Y cells, electron acceptor function in the oxidation of NADH by *Clostridium kluyveri* diaphorase or rat liver mitochondria and inhibition against avian myeloblastosis virus reverse transcriptase. The significantly lower cytotoxicity of sakyomicin A as compared with juglone was attributable to its poor membrane transport. The inhibition of reverse transcriptase activity may result from the interaction between a sulfhydryl group in the active center of the enzyme and quinone groups of the naphthoquinones and sakyomicin A.

Retroviruses contain an enzyme called reverse transcriptase, which is responsible for the first step of the integration of viral genomes into cellular DNA, and agents which inhibit this enzyme are therefore of potential therapeutic use against viral infection.

In the course of our screening for the enzyme inhibitors against reverse transcriptase of avian myeloblastosis virus (AMV), novel substances, retrostatin[1] and chromostin[2], and limocrocin[3] which had been reported as a pigment produced by *Streptomyces limosus*[4] were isolated as the specific enzyme inhibitors. Furthermore, one of the active metabolites of *Streptomyces* origin was proven to be identical with sakyomicin A[5], an antibiotic produced by a strain of *Nocardia*[6]. Besides an inhibitory activity against reverse transcriptase[5], sakyomicin A showed an electron acceptor activity in the oxidation of NADH by bacterial diaphorase or mitochondria[7]. Similar to streptonigrin which is another potent inhibitor of reverse transcriptase[8,9], only a catalytic amount of sakyomicin A was necessary for the oxidation of NADH, though exogenous NADH was oxidized by mitochondria in the presence of sakyomicin A but not streptonigrin. In accordance with the oxidation of NADH, sakyomicin A was reduced to a hydroquinone which, in turn, autoxidized to a quinone accompanying the generation of hydrogen peroxide. In contrast to streptonigrin which had been reported to primarily interfere with oxidative phosphorylation in mitochondria by facilitating the oxidation of NADH by DT-diaphorase[10~12], the enzyme involved in sakyomicin A-dependent oxidation of NADH appeared to be in a different compartment in mitochondria from DT-diaphorase.

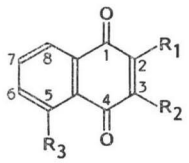
The present study was undertaken to examine the role of the naphthoquinone moiety in the biological activities of sakyomicin A using various naphthoquinone derivatives.

Materials and Methods

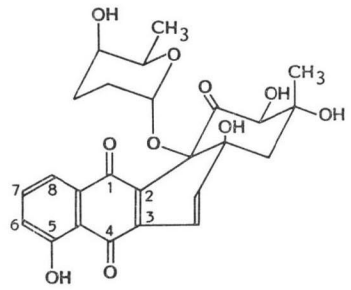
Materials

Sakyomicin A was kindly donated by Prof. N. TANAKA, Institute of Applied Microbiology, Uni-

Fig. 1. Structures of the naphthoquinones and sakyomicin A.



Compound	R_1	R_2	R_3
1	H	H	H
2	H	H	OH
3	Cl	Cl	H
4	CH_3	H	H
5	OH	H	H
6	OH	$(CH_2)_2COOH$	H
7	OH	$(CH_2)_2COOEt$	H



Sakyomicin A

versity of Tokyo. 1,4-Naphthoquinone (**1**) was obtained from Tokyo Kasei Kogyo Co., Ltd., Tokyo. Juglone (5-hydroxy-1,4-naphthoquinone, **2**), 2,3-dichloro-1,4-naphthoquinone (**3**) and menadione (2-methyl-1,4-naphthoquinone, **4**) were products of Nakarai Chemicals Ltd., Kyoto. 2-Hydroxy-1,4-naphthoquinone (**5**) was purchased from Aldrich Chemical Co., Inc., Wis. 3-(2-Carboxyethyl)-2-hydroxy-1,4-naphthoquinone (**6**) and 3-(2-ethoxycarbonylethyl)-2-hydroxy-1,4-naphthoquinone (**7**) were prepared according to the methods of PETTIT and HOUGHTON[13], and FIESER and LEFFLER[14]. Diaphorase from *Clostridium kluyveri* was purchased from Oriental Yeast Co., Ltd., Tokyo. All other materials were commercial products of the analytical grade.

Assay Methods for Biological Activities

The detailed assay methods for reverse transcriptase and DNA-directed DNA polymerase I of *Escherichia coli* were described in the previous papers[3,8].

Growth inhibitory activity against murine lymphosarcoma L5178Y cells was determined as described previously[15].

Determination of Hydrogen Peroxide

Hydrogen peroxide was measured by the oxidative coupling of 4-aminoantipyrine with phenol by horse radish peroxidase to produce a quinone-imine dye with an absorption maximum at 500 nm[16]. The details were described in the previous paper[7].

Oxidation of NADH or NADPH by Rat Liver Mitochondria

Rat liver mitochondria were isolated by the method of JOHNSON and LARDY[17] and protein concentrations were determined by the method of LOWRY *et al.*[18]. The oxidation of NADH or NADPH by mitochondria was measured at 30°C by oxygen consumption as determined with a Clark type electrode (Yellow Spring Instrument Co., Yellow Spring, Ohio)[19].

Respiration of Rat Liver Mitochondria

Respiration of mitochondria on glutamate was measured by the same method as described in the measurement of the oxidation of NADH or NADPH.

Results

The activity of AMV reverse transcriptase was markedly inhibited by **1**, **2**, **3** or **4** at 40 μg/ml, while moderate inhibition was observed by sakyomicin A and no significant inhibition by either **5**, **6** or **7** at the same concentration (Table 1). **2** was the most active among the naphthoquinones tested with more than 80% inhibition even at 10 μg/ml. On the other hand, ID_{50} of **1** and **2** against *E. coli* DNA-directed DNA polymerase I were approximately 80 μg/ml.

As for cytotoxicity against L5178Y cells, ID_{50} of **1**, **2**, **3**, **4** and sakyomicin A were 0.089, 0.024, 0.14,

Table 1. Effects of the naphthoquinones and sakyomicin A on AMV reverse transcriptase.

Compound	Concentration (μg/ml)	Inhibition (%)
1	40	85
	10	50
2	40	94
	10	82
3	40	76
	10	34
4	40	77
	10	32
5	40	0
	10	0
6	40	0
	10	0
7	40	0
	10	0
Sakyomicin A	40	63
	10	26

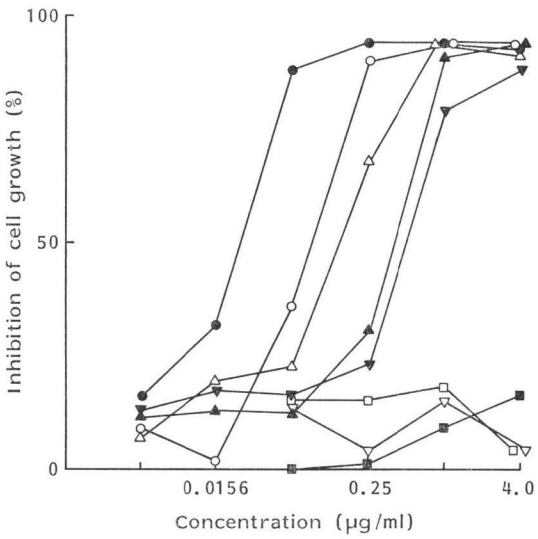
Fig. 2. Effects of the naphthoquinones and sakyomicin A on the growth of L5178Y cells.

A test sample dissolved in DMSO at 2.5 mg/ml was diluted with serum-free Fischer's medium to make a test solution.

A mixture of the test solution (0.2 ml) and the cell suspension ($5.0\sim6.0\times10^4$ cells/1.8 ml) in Fischer's medium containing 10% horse serum (Grand Island Biological Co.) was incubated in a tightly capped test tube at 37°C for 72 hours[15].

The cell numbers were determined in a Model Dn Coulter counter.

1 ○, **2** ●, **3** △, **4** ▲, **5** □, **6** ■, **7** ▽, sakyomicin A ▼.



0.39 and 0.48 μg/ml, respectively, and those of **5**, **6** and **7** were much higher than 4.0 μg/ml (Fig. 2).

Generation of hydrogen peroxide in conjunction with the oxidation of NADH or NADPH by *C. kluyveri* diaphorase is shown in Fig. 3. Hydrogen peroxide was produced in the presence of a catalytic amount of **1**, **2**, **3** or **4**, as was the case with sakyomicin A, whereas no significant effect on the generation of hydrogen peroxide was observed in the presence of **5**, **6** or **7** even at the highest concentration tested. **2** was the most active among the naphthoquinones and the activities of **3** and **4** were comparable to that of **1**. In general, NADPH was more preferably oxidized by bacterial diaphorase than NADH except for the case of **3**.

Oxygen consumption coupled to the oxidation of NADH or NADPH by isolated rat liver mitochondria is shown in Fig. 4. In well accordance with the results for sakyomicin A, significant enhancement in the oxidation of NADH but not NADPH was observed by adding either **1**, **2** or **3** to the reaction medium. Though **4** was one of the potent electron acceptors in the oxidation of NADH by bacterial diaphorase, the oxidation of NADH by mitochondria in the presence of **4** was not remarkable.

The effects of the naphthoquinones and sakyomicin A on the oxidation of glutamate by mitochondria are shown in Fig. 5. In contrast to the pattern obtained with sakyomicin A, a rapid but not continuous decline in oxygen content was observed in the presence of **1** or **2**. A steady but not sharp decline was, however, observed in the presence of **4**. Again, **5**, **6** and **7** exerted no effect on the respiration of mitochondria.

Discussion

The results presented in this report support the conclusion that the naphthoquinone moiety is es-

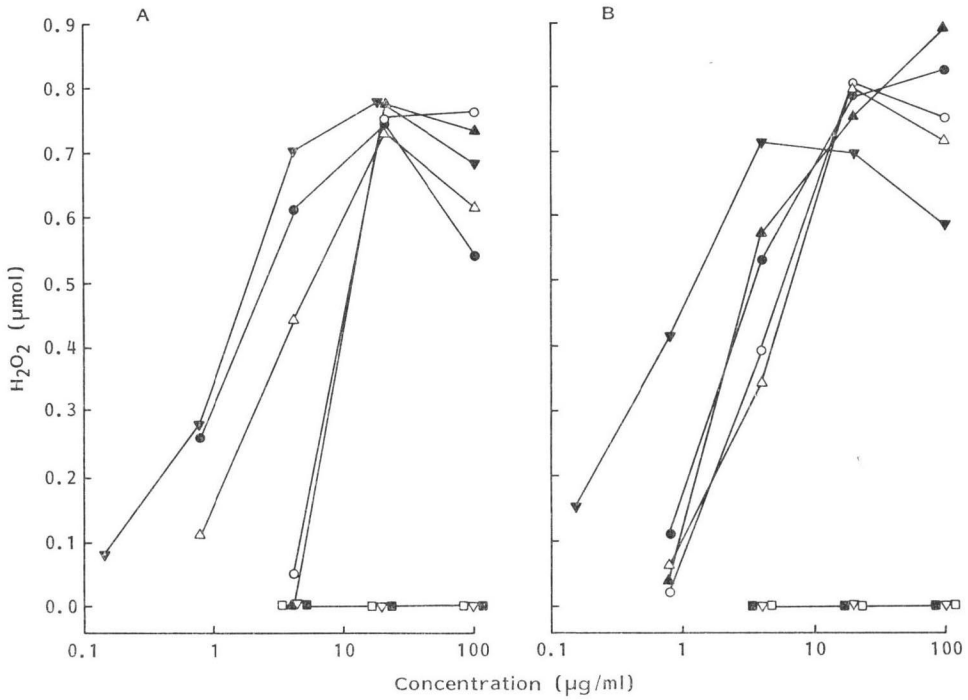
Fig. 3. Generation of hydrogen peroxide coupled to the oxidation of NADH or NADPH by *C. kluyveri* diaphorase.

A mixture of 9.0 units/ml diaphorase, 0.9 mM NADH (A) or NADPH (B), 0.05 mM EDTA and 10 mM Tris-HCl (pH 8.0) was incubated with a test sample in a final volume of 1 ml at 37°C for 5 minutes.

Hydrogen peroxide formed was measured by the method described previously[7].

Test samples were dissolved in and diluted with DMSO to adjust the final concentration of DMSO at 1%.

1 ○, **2** ●, **3** △, **4** ▲, **5** □, **6** ■, **7** ▽, sakyomicin A ▼.



sential to the biological activities of sakyomicin A. Furthermore, the hydroxyl group at C-5 of this moiety might have some responsibility for the enhanced electron acceptor activity of sakyomicin A as suggested by the highest activity of **2** among the naphthoquinones tested. The methyl group at C-2 makes **4** unique; in marked contrast to the result obtained with **1**, **2** or **3**, no enhanced oxidation of NADH by mitochondria was observed in the presence of **4** as can be seen in Fig. 4. By this fact, it is partially accounted for that **4** is much less cytotoxic against L5178Y cells as compared with **1** and **3** which are as potent electron acceptors as **4** in the oxidation of NADH by bacterial diaphorase. The hydroxyl group at C-2 might abolish the biological activities of **5**, **6** and **7** which are inactive in cytotoxicity against L5178Y cells, enhancement of the oxidation of NADH by bacterial diaphorase or mitochondria and inhibition against reverse transcriptase. Although no marked activity was manifested by **6** or **7**, the adverse effect of the hydroxyl group at C-2 had been reported to be antagonized by appropriate side chains at C-3[20]. Since the electron acceptor activities of the naphthoquinones are well correlated with their inhibitory activities against reverse transcriptase, a sulfhydryl group in the catalytic center of reverse transcriptase is considered to be highly reactive with their quinone groups as speculated by the others[21]. This is further confirmed by the fact that **1**, **2**, **3** and **4** also inhibited the activity of *Alcaligenes* creatine amidinohydrolase (creatinase, EC 3.5.3.3)[22], another sulfhydryl enzyme, at the same range of drug concentrations (unpublished observation). The bulky structure of sakyomicin A as compared with those of the naphthoquinones might disturb its access to the catalytic center of reverse transcriptase, resulting in a decreased inhibition of the enzyme activity.

The effects of the naphthoquinones on the respiration of mitochondria using glutamate as a sub-

Fig. 4. Effects of the naphthoquinones and sakyomicin A on the oxidation of NADH or NADPH by rat liver mitochondria.

The oxidation of NADH (A) or NADPH (B) by rat liver mitochondria was measured in 3 ml of a basal medium consisting of 225 mM sucrose, 5 mM potassium phosphate and 10 mM Tris-HCl (pH 7.4).

Test samples were dissolved in DMSO at 5 mg/ml and used at the doses indicated in the figure.

The other additions were as follows (stock solution): Mitochondria (M), 1.5 mg as protein; NADH (100 mM in H_2O), 1.0 μmol; NADPH (100 mM in H_2O), 1.0 μmol.

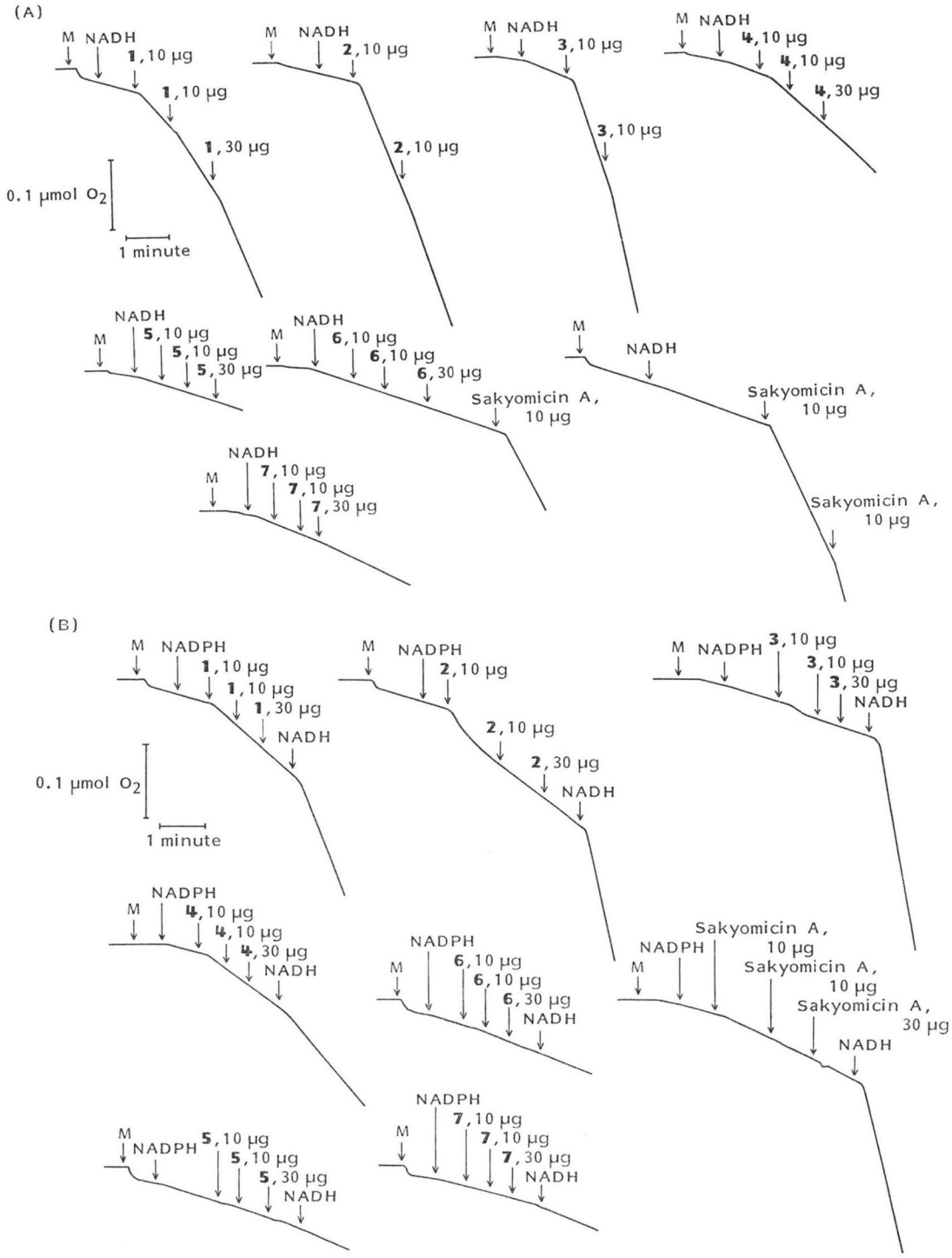
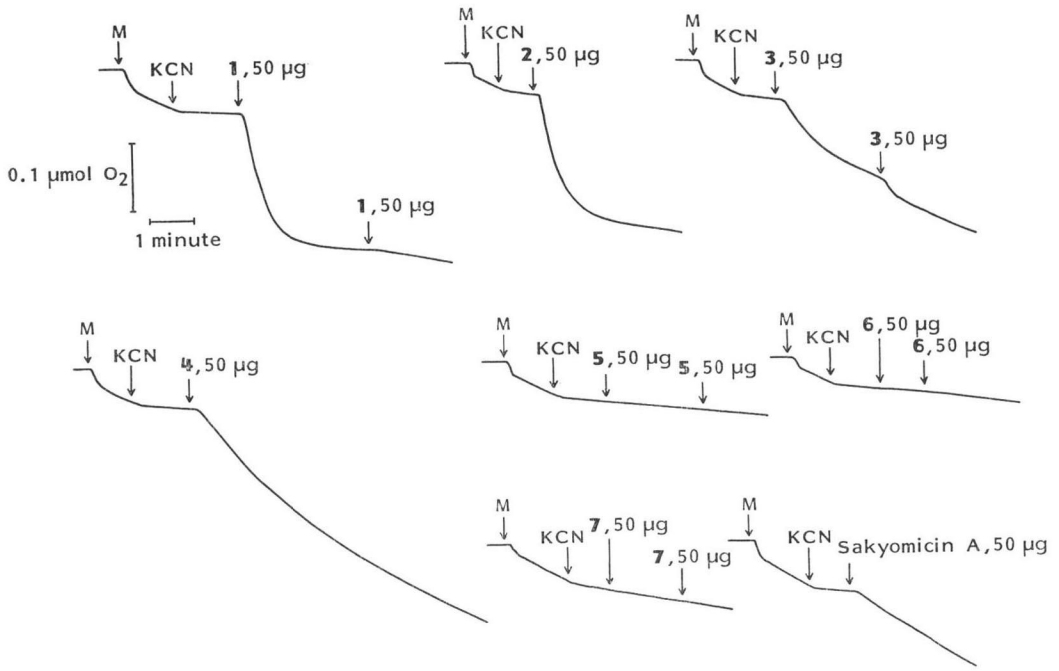


Fig. 5. Effects of the naphthoquinones and sakyomicin A on the oxidation of glutamate by rat liver mitochondria.

Respiration of mitochondria was measured in 3 ml of the basal medium supplemented with 15 mM glutamate at 30°C.

Test samples were dissolved in DMSO at 5 mg/ml and used at the doses indicated in the figure.

The other additions (stock solution): Mitochondria (M), 1.5 mg as protein; KCN (100 mM in H_2O), 1 μmol.



strate were investigated along with that of sakyomicin A. Soon after the addition of **1** or **2**, oxygen consumption in a rapid but not continuous fashion was observed. In contrast, only a trace activity was observed in the presence of sakyomicin A, suggesting its poor membrane transport in mitochondria which might account for a very low cytotoxicity against L5178Y cells. This was beneficial when sakyomicin A was used as an antiviral agent against the *in vitro* infection of AIDS virus (HTLV-III) as described elsewhere[5].

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