MECHANISM OF INHIBITION OF REVERSE TRANSCRIPTASE BY QUINONE ANTIBIOTICS

II. DEPENDENCE ON PUTATIVE QUINONE POCKET ON THE ENZYME MOLECULE

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Inhibition of avian myeloblastosis virus (AMV) reverse transcriptase by natural and synthetic quinones including antibiotics could be accounted for by an oxidation-reduction reaction. The quinones were shown to function as electron acceptors as revealed by the catalytic oxidation of NADH by *Clostridium kluyveri* diaphorase which was in excellent agreement with enzyme inhibition activity.

The kinetics of inhibition of AMV reverse transcriptase by three synthetic quinones with different core structures, *i.e.*, 6-methoxy-5,8-dihydroquinoline-5,8-dione, 5,8-dihydroisoquinoline-5,8-dione and 1,4-naphthoquinone, were studied. These quinones inhibited reverse transcriptase in the same manner as streptonigrin (STN) and were shown to act at a single class of reaction site(s) on the enzyme molecule. In contrast, the quinones with bulky substituents, *i.e.*, 7-(2-nitrophenethylamino)-5,8-dihydroisoquinoline-5,8-dione and 7-methoxy-6-methyl-3-piperidino-5,8-dihydroisoquinoline-5,8-dione, were inactive as inhibitors of reverse transcriptase, whereas they retained competent catalytic activities in the oxidation of NADH by *C. kluyveri* diaphorase. Based on these observations, the existence of a specific site of interaction on the enzyme molecule, referred to as a quinone pocket, was proposed. The quinone pocket might play a crucial role in the early sequence of events leading to the inhibition of reverse transcriptase by quinones including STN and sakyomicin A (SKM). Access of SKM to a quinone pocket might be restricted due to its bulky structure in the vicinity of the quinone group. This is inferred from unsuccessful inhibition of reverse transcriptase by the quinones with bulky substituents, resulting in much poorer inhibition of reverse transcriptase in spite of more potent electron acceptor activity in the oxidation-reduction system as compared with those of STN.

Streptonigrin (STN) was first reported as an antitumor antibiotic by RAO and CULLEN[1] Later, potent inhibition of avian myeloblastosis virus (AMV) reverse transcriptase by STN was observed by CHIRIGOS *et al.*[2].

STN showed specificity for AMV reverse transcriptase in comparison with calf thymus DNA-dependent DNA polymerase (DDDP) alpha and *Escherichia coli* DDDP I[3]. By using various quinones, the importance of the role of the quinone group in the biological activities of STN and good correlation between electron acceptor activities in the oxidation of NADH by *Clostridium kluyveri* diaphorase and inhibitory activities against AMV reverse transcriptase were reported previously[4~6]. Depending on these observations, the specificity shown by STN are considered to derive from the

high sensitivity of AMV reverse transcriptase to the oxidation-reduction reaction mediated by quinones including STN.

Inhibition of AMV reverse transcriptase by STN was characterized to be noncompetitive with respect to substrate and might depend on direct interaction between STN and the enzyme molecule or be mediated by molecular oxygen[7,8]. The combined addition of NADH and diaphorase to the enzyme assay system, however, could not afford any effect on the inhibition of AMV reverse transcriptase by the quinone antibiotics, STN and sakyomicin A (SKM)[9]; the latter is another type of quinone antibiotic showing inhibition of AMV reverse transcriptase. SKM was more potent as an electron acceptor in NADH/*C. kluyveri* diaphorase system but less active as an inhibitor of reverse transcriptase when compared with STN[9]. In this paper, the kinetics of inhibition of AMV reverse transcriptase and the biological properties of the synthetic quinones were studied in an attempt to find clues to these questions.

Materials and Methods

Materials

Isolation of STN was conducted following the previously reported method[10]. Mimosamycin (**7**), an antibiotic produced by *Streptomyces lavendulae* No. 314, was obtained as reported previously[11,12]. The details of preparation of 6-methoxy-5,8-dihydroquinoline-5,8-dione (**1**), 5,8-dihydroisoquinoline-5,8-dione (**2**), 7-hydroxy-6-methyl-3-piperidino-5,8-dihydroisoquinoline-5,8-dione (**5**), 7-methoxy-6-methyl-3-piperidino-5,8-dihydroisoquinoline-5,8-dione (**6**), 5-methoxy-1,6-dimethyl-7,8-dihydroisoquinoline-7,8-dione (**8**), 1-cyano-5-methoxy-6-methyl-7,8-dihydroisoquinoline-7,8-dione (**9**), 5,8-diacetoxy-7-methoxy-6-methyl-3-piperidinoisoquinoline (**10**) were reported previously[12~17]. The synthesis of 7-amino-5,8-dihydroisoquinoline-5,8-dione (**3**), 7-(2-nitrophenethylamino)-5,8-dihydroisoquinoline-5,8-dione (**4**), 2-ethyl-1,4-naphthoquinone (**14**) and 2-amino-1,4-naphthoquinone (**15**) will be published elsewhere. 1,4-Naphthoquinone (**11**) was purchased from Tokyo Kasei Kogyo Co., Ltd. 2-Amino-3-chloro-1,4-naphthoquinone (**17**) was a product of Aldrich Chemicals., Inc. Vitamin K_1 (**18**) was obtained from Nakarai Chemicals Ltd. Reverse transcriptase from AMV was purchased from Seikagaku Kogyo Co., Ltd. Diaphorase of *C. kluyveri* origin was a product of Oriental Yeast Co., Ltd. Poly(rA) and oligo$(dT)_{12-18}$ were obtained from Sigma Chemical Co., Ltd. and Pharmacia (Japan) Kabushiki Kaisha, respectively. All other chemicals were commercial products of analytical grade.

Assay Methods for Biological Activities

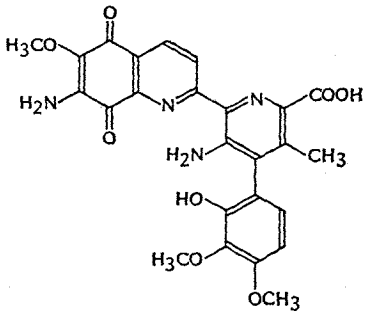
The details of assay method for AMV reverse transcriptase were described previously[3]. Briefly, standard assay solution containing 50 mM Tris-HCl (pH 8.0), 2.5 mM $MgCl_2$, 2.5 mM dithiothreitol, 30 mM NaCl, 100 μM thymidine-5'-triphosphate (TTP), 6 μCi/ml [^{3}H]TTP, 5 μg/ml poly(rA), 0.25 μg/ml oligo$(dT)_{12-18}$ and 3.0 U/ml reverse transcriptase in a final volume of 100 μl was incubated at 37°C for 30 minutes, unless otherwise specified. The rate of incorporation of [^{3}H]TTP into high molecular fractions was proportional to the reaction time up to 30 minutes.

The method for culture of murine lymphoblastoma L5178Y cells was described in a previous paper[18].

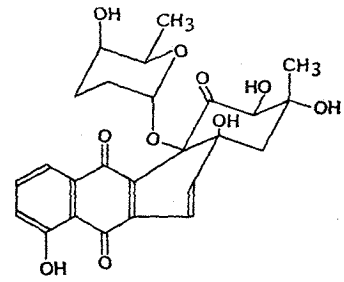
Determination of Hydrogen Peroxide

Hydrogen peroxide was determined by the method of TRINDER[19] with some modifications as described in a previous paper[9]. The reaction mixture (2.5 ml) consisting of 10 mM Tris-HCl (pH 8.0), 0.54 mM 4-aminoantipyrine, 0.006% phenol and 6 U/ml peroxidase was incubated at 37°C for 20 minutes.

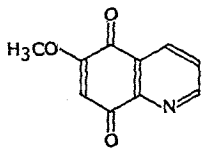
Fig. 1. Structures of streptonigrin, sakyomicin A and the quinones.



Streptonigrin



Sakyomicin A



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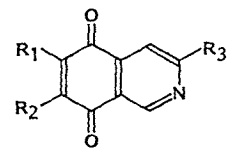
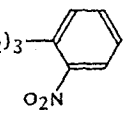


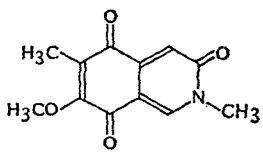
2 $R_1 = R_2 = R_3 = H$

3 $R_1 = R_3 = H$ $R_2 = NH_2$

4 $R_1 = R_3 = H$ $R_2 = NH(CH_2)_3$ 

5 $R_1 = CH_3$ $R_2 = OH$ $R_3 =$ 

6 $R_1 = CH_3$ $R_2 = OCH_3$ $R_3 =$ 



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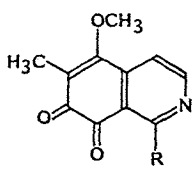
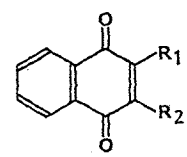


8 $R = CH_3$

9 $R = CN$



11 $R_1 = R_2 = H$

12 $R_1 = OH$ $R_2 = H$

13 $R_1 = CH_3$ $R_2 = H$

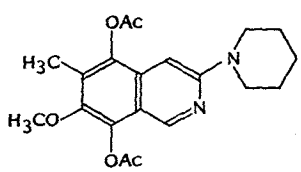
14 $R_1 = C_2H_5$ $R_2 = H$

15 $R_1 = NH_2$ $R_2 = H$

16 $R_1 = R_2 = Cl$

17 $R_1 = NH_2$ $R_2 = Cl$

18 $R_1 = CH_3$ $R_2 = C_{20}H_{39}$



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Results

Enzyme activity was measured in the presence of different concentrations of individual components of reaction mixture, while the other components were kept at the same concentrations as those in the standard reaction mixture. The results summarized in Table 1 demonstrate that inhibition of reverse transcriptase was most efficiently reversed by increasing the concentration of enzyme. In contrast, increase in the concentration of poly(rA)-oligo(dT)$_{12-18}$ was accompanied by marginal enhancement in inhibition of reverse transcriptase by **1**, **2** or **11**, whereas the concentration of TTP had no effect on inhibition of reverse transcriptase.

Incubation of reverse transcriptase with **1**, **2** or **11** in advance of the constitution of a complete reaction mixture potentiated inhibition of reverse transcriptase (Fig. 2).

Table 1. Inhibition of AMV reverse transcriptase by **1**, **2** and **11**.

(A) Effect of the concentration of enzyme.

Enzyme (U/ml)	Inhibition (%)		
	1 (2.5 μg/ml)	**2** (0.31 μg/ml)	**11** (0.62 μg/ml)
6.0	66.7	74.2	43.5
3.0	84.4	81.4	81.9
1.5	100	100	95.6
0.75	100	100	100

(B) Effect of the concentration of TTP.

TTP (μM)	Inhibition (%)		
	1 (2.5 μg/ml)	**2** (0.31 μg/ml)	**11** (0.62 μg/ml)
100	66.1	65.6	71.0
50	57.5	NT	69.1
25	68.6	59.3	69.2
12.5	57.5	NT	75.0
6.25	70.3	67.1	70.5

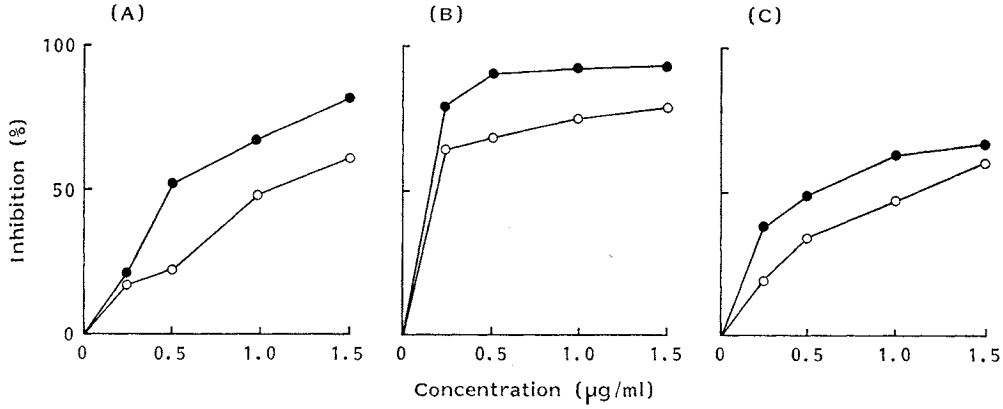
(C) Effect of the concentration of template-primer.

Poly(rA) (μg/ml)	Oligo(dT)$_{12-18}$ (μg/ml)	Inhibition (%)		
		1 (2.5 μg/ml)	**2** (0 31 μg/ml)	**11** (0.62 μg/ml)
80	4.0	84.3	70.6	66.8
40	2.0	79.5	74.3	58.8
20	1.0	71.3	63.3	48.5
10	0.50	56.8	53.8	51.1
5	0.25	48.2	43 0	42.3

A reaction mixture in a final volume of 100 μl was incubated at 37°C for 30 minutes. After the reaction was terminated by cooling in an ice bath, 50-μl aliquot was soaked into a 2.4 cm-round piece of DEAE-cellulose paper which was washed three times with 5% $Na_2HPO_4 \cdot 12H_2O$ and once each with distilled water and ethanol. The radioactivities of [^{3}H]TTP incorporated in the absence (a) and presence (b) of the individual quinones were used to calculate inhibition (%) which was defined in the following equation: Inhibition (%)$=(1-b/a)\times 100$. The quinones were used at the concentrations which were expected to give approximately 50% inhibition under the standard reaction conditions (refer to Table 2).

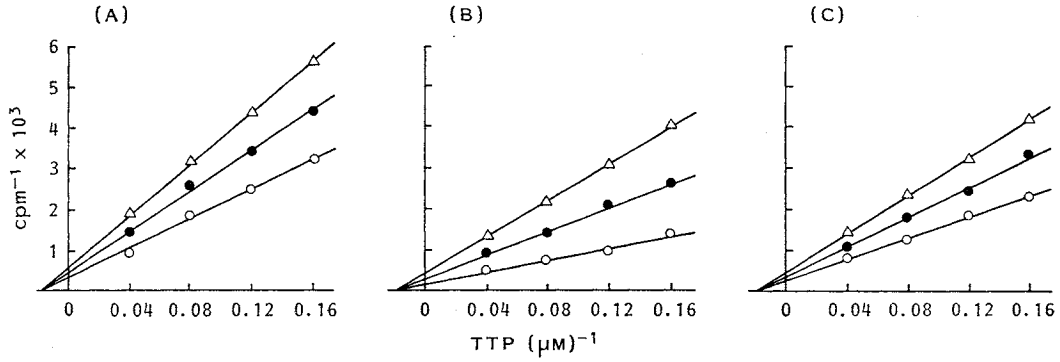
NT: Not tested.

Fig. 2. Effect of preincubation of AMV reverse transcriptase with the quinones.
(A) **1**, (B) **2**, (C) **11**.



The enzyme was incubated in the absence (○) or the presence (●) of individual quinones at 27°C for 12 minutes in advance of the constitution of a reaction mixture. The whole was further incubated at 37°C for 30 minutes.

Fig. 3. Lineweaver-Burk plot of AMV reverse transcriptase activity as a function of TTP concentration.



(A) The initial rate of [^{3}H]TTP incorporation was measured in the absence (○) or presence of 1.25 (●) or 2.5 μg/ml (△) of **1**.
(B) The initial rate of [^{3}H]TTP incorporation was measured in the absence (○) or presence of 0.31 (●) or 0.62 μg/ml (△) of **2**.
(C) The initial rate of [^{3}H]TTP incorporation was measured in the absence (○) or presence of 0.5 (●) or 1.0 μg/ml (△) of **11**.

The initial rate of incorporation of [^{3}H]TTP was measured in the absence or the presence of **1**, **2** or **11**. The Lineweaver-Burk plots for **1**, **2** and **11** shown in Fig. 3 are characteristic of noncompetitive inhibition with respect to TTP; *i.e.*, decreased Vmax and unaltered *Km* values for [^{3}H]TTP incorporation. In contrast, the kinetic parameters obtained as a function of the concentration of template-primer indicate that inhibition of reverse transcriptase was uncompetitive (data not shown).

The dose-response data were constructed in modified Scatchard plots (Fig. 4). From the results, the existence of a single class of reaction site(s) on the enzyme molecule was evident for all the quinone compounds tested including STN.

The effects of quinones on [^{3}H]TTP incorporation in an ongoing reaction are shown in Fig. 5.

In all cases, the rate of [^{3}H]TTP incorporation was inhibited following a short time lag. The results indicate that all the quinones inhibit DNA synthesis at the elongation step as in the case of STN[7].

Comparative biological properties of various quinone antibiotics and synthetic compounds are shown in Table 2. The data for STN, SKM, **1**, **11**, **12**, **13** and **16** are cited from previous reports[3,4,6,20,21]. The existence of the hydroxyl group at *ortho*-position to the carbonyl group on the quinone ring, as in the case of **5** or **12**, nullified all biological activities and the requirement for a role of the quinone group in effective inhibition of reverse transcriptase and electron acceptor function coupled with the oxidation of

Fig. 4. Modified Scatchard plot of dose-response data in inhibition of AMV reverse transcriptase by the quinones and STN.

○ **1**, ● **2**, △ **11**, ▲ STN.

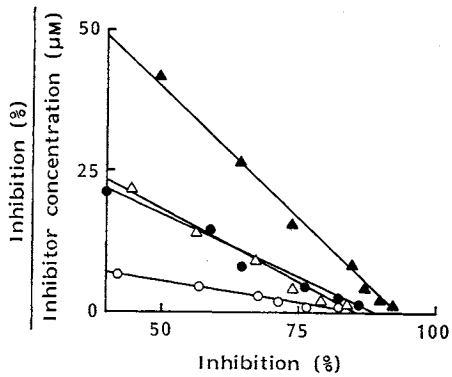
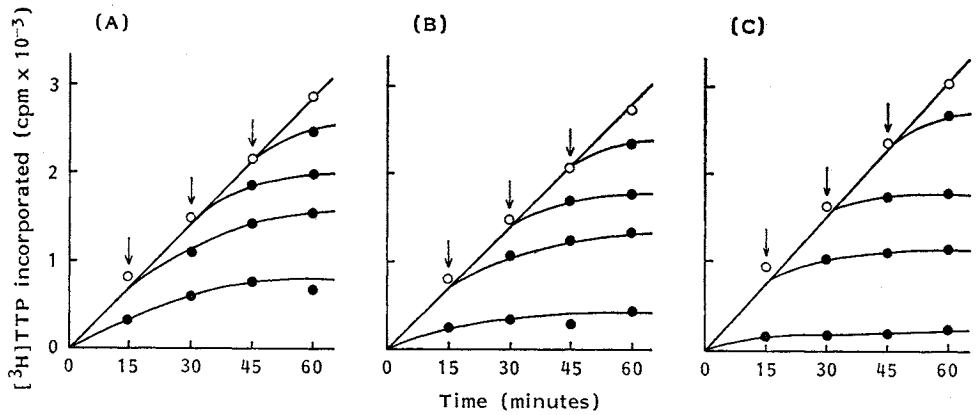


Table 2. Comparison of biological activities of STN and, SKM and the quinones.

Compound	ID_{50} (μg/ml)		ED_{50} (μg/ml)[b]
	RT (AMV)[a]	L5178Y	H_2O_2
STN	3.0	0.0025	38
SKM	27	0.51	7.0
1	2.3	0.21	3.6
2	0.5	0.33	5.8
3	1.8	0.098	<0.8
4	>40	1.7	12
5	>40	>4	>100
6	>40	>4	9.2
7	35	2.1	55
8	7.3	0.98	4.3
9	1.9	0.72	4.1
10	>40	2.6	>100
11	0.63	0.089	9.8
12	>40	>4	>100
13	0.82	0.39	6.6
14	4.9	NT	48
15	15	2.2	33
16	17	0.14	5.5
17	35	>4	32
18	>40	NT	>100

[a] AMV reverse transcriptase.
[b] The concentration to give 50% the maximum effect[9].
NT: Not tested.

Fig. 5. Effect of the quinones on ongoing AMV reverse transcriptase reaction.
(A) **1**, (B) **2**, (C) **11**.



The quinones were added to a reaction mixture at the times indicated by the arrows at the final concentration of 2.5 μg/ml.

NADH by *C. kluyveri* diaphorase was shown by the results for **7**. *para*-Isoquinoline quinones with rather bulky substituent, **4** and **6**, were devoid of inhibitory activity against reverse transcriptase, although their activities as electron acceptors were comparable to those of the other compounds. Electron acceptor and cytocidal activities of **3** were higher than those of **2** owing to the contribution of the amino group at C-7 on the 5,8-dihydroisoquinoline-5,8-dion structure. In contrast, the amino group at C-2 on the 1,4-naphthoquinone structure had adverse effect on these activities of naphthoquinones as can be deduced from the results for **11**, **15**, **16** and **17**, whereas reverse transcriptase was inhibited by them to the same extents. Regarding the alkyl substituents at C-2 and/or C-3 on the 1,4-naphthoquinone structure, chain elongation was inversely correlated with biological activities as exemplified by the results for **13**, **14** and **18**.

Discussion

The specificity of STN for AMV reverse transcriptase elicited our great interest in the elucidation of mechanisms by which STN interferes with the enzyme reaction. The quinoline quinone moiety of STN was shown to play an important role in inhibition of AMV reverse transcriptase as seen by examination of comparative biological properties of two quinoline quinone derivatives and STN[4]. However, it remained unsolved whether the quinones inhibited AMV reverse transcriptase in a similar manner to STN.

The synthetic quinones, **1**, **2** and **11**, inhibited AMV reverse transcriptase due to direct interaction with the enzyme molecule in a noncompetitive manner with respect to substrate as in the case of STN. The uncompetitive inhibition with respect to template-primer suggests that the enzyme becomes more susceptible to quinone-dependent inactivation provided that it forms a complex with template-primer, resulting in an enhanced inactivation of the enzyme at the higher concentrations of template-primer (Table 1). The results shown in Fig. 4 suggest the existence of a single class of interaction site(s) on the enzyme molecule for all the quinones and STN. The lack of inhibitory activity against AMV reverse transcriptase in the quinones with rather bulky substituent such as **4** and **6** inspite of their competence as electron acceptors is similar to the discrepancy observed between those activities of SKM. The previous observation that quinone antibiotic-induced inhibition of AMV reverse transcriptase was not potentiated by the supplementation of NADH and *C. kluyveri* diaphorase to the reaction mixture[9] excludes the possibility that a series of events resulting in inhibition of reverse transcriptase are mediated by molecular oxygen, supporting the postulation of a quinone pocket, which in turn gave STN specificity for AMV reverse transcriptase.

Acknowledgment

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