Notes

ROLE OF SINGLE-ELECTRON REDUCTION POTENTIAL IN INHIBITION OF REVERSE TRANSCRIPTASE BY STREPTONIGRIN AND SAKYOMICIN A

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Streptonigrin was first isolated from *Streptomyces flocculus* as an antitumor antibiotic[1]. Later, the antibiotic was recognized to be a potent inhibitor of avian myeloblastosis virus (AMV) reverse transcriptase[2]. Recently, we reported that streptonigrin inhibited AMV reverse transcriptase mainly by interacting with an enzyme molecule[3]. According to the results of the kinetic analysis, streptonigrin inhibited reverse transcriptase in a non-competitive manner. As reported previously[4~6], the cytotoxicity of streptonigrin results in part from its bypassing effect on the mitochondrial oxidative phosphorylation. In the presence of catalytic amounts of streptonigrin, DT-diaphorase catalyzes the oxidation of NADH supplied by the mitochondrial redox system. The hydroquinone of streptonigrin formed in conjunction with the oxidation of NADH in turn autoxidizes to the quinone by transferring electrons to molecular oxygen. The electron-acceptor activity of streptonigrin was also observed in the oxidation of NADH by *Clostridium kluyveri* diaphorase[6]. Like streptonigrin, sakyomicin A is a quinone antibiotic with an inhibitory activity against reverse transcriptase[7,8], though the latter is less active than the former. Sakyomicin A played the role of electron acceptor in the oxidation of NADH by both mitochondrial and *C. kluyveri* diaphorase, as in the case of streptonigrin[8,8]. Further, we observed a close correlation between the electron-acceptor function and the inhibitory activity against reverse transcriptase using the naphthoquinone derivatives[8]. Segregation of the cytotoxicity from the enzyme inhibitory activity has been demonstrated for the quinoline quinone compounds[9,10].

WICK and FITZGERALD suggested that the inhibition of AMV reverse transcriptase by quinol and quinone compounds such as levodopa and 1,4-benzoquinone was manifested by the common intermediate of oxidation of quinols or reduction of quinones, *i.e.*, semiquinone[11]. The semiquinone which results from the addition of single-electron to the quinone or loss of an electron from the quinol may act either as an oxidizing or reducing agent with transfer of its electron to substrate or acceptance of a second electron with formation of a quinol or quinone, respectively.

As described above, both sakyomicin A and streptonigrin were active as electron acceptors in the oxidation of NADH by *C. kluyveri* diaphorase. The activity was determined by an amount of hydrogen peroxide which was generated by either two-electron or single-electron reduction of molecular oxygen. The former probably results from the donation of two electrons from the autoxidizing hydroquinone to molecular oxygen, and in the latter case, the semiquinone may exist as an intermediate which accepts a single-electron from NADH and donates an electron to molecular oxygen, resulting in the generation of superoxide anion (O_2^-). In terms of the specific activity in the inhibition of reverse transcriptase, streptonigrin was 10-fold more active than sakyomicin A; the ID_{50} values were 51 μM for sakyomicin A and 5.9 μM for streptonigrin. It seemed to be interesting to compare the contributions of two-electron and single-electron reductions of molecular oxygen to the generation of hydrogen peroxide, in other words, the one-electron reduction potential of individual antibiotics and quinones.

Ferricytochrome c (horse heart, Type III), catalase (bovine liver, EC 1.11.1.6) and NADH were purchased from Sigma Chemical Co.

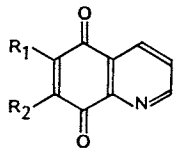
Superoxide anion dismutase (SOD, bovine erythrocytes, EC 1.15.1.1) was a product of Miles Lab., Inc. Diaphorase (*C. kluyveri*, EC 1.6.99.) was obtained from Oriental Yeast Co., Ltd. All other chemicals were the commercial products of the analytical grade. The activity of *C. kluyveri* diaphorase was measured by the generation of hydrogen peroxide as described previously[6]. The O_2^- generating activity was measured by the SOD-inhibitable ferricytochrome c assay. Briefly, a mixture of 0.92 unit/ml *C. kluyveri* diaphorase, 14 units/ml of catalase, 75 units/ml SOD, 100 μM EDTA, 100 μM NADH, 50 μM ferricytochrome c and 8 mM PIPES buffer (pH 7.3) in a final volume of 2.5 ml was incubated at 37°C for 10 minutes, and in the meantime the absorbance at 550 nm was read against the blank, from which SOD and catalase were removed, using the millimolar absorption coefficient of 21.1. Although *C. kluyveri* diaphorase mediates oxygen-independent reduction of ferricytochrome c, oxygen-dependent one, which is sensitive to SOD and catalase, dominates the reduction of ferricytochrome c in the presence of the quinones. The results are shown in Table 1. The A/B ratios are normalized based on the concentration of diaphorase, where the A/B of *ca.* 1 indicates the exclusive single-electron reduction. Unexpectedly from the inhibitory activities of these two antibiotics against reverse transcriptase, the single-electron reduction of molecular oxygen prevails in the presence of sakyomicin A, while the contribution of the same reaction is approximately 5% in the case of streptonigrin. The lack of correlation between the reverse transcriptase inhibitory activity and the single-electron reduction potential was also observed by the synthetic heterocyclic quinones and naphthoquinones. Furthermore, the cytotoxicity of individual antibiotics and quinones could not be predicted from the electron-acceptor activity

Table 1. Comparison of biological properties of streptonigrin, sakyomicin A and quinones.

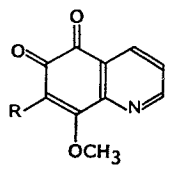
Compound	ID_{50} (μM)[8~10]		ED_{50} (μM)[a]		A/B modified[d]
	L5178Y[b]	RDDP[c]	H_2O_2 (A)	O_2^- (B)	
Streptonigrin	0.005	5.9	76	140	0.055
Sakyomicin A	0.99	51	14	1.3	1.1
1	2.0	13	17	46	0.038
2	1.7	16	16	27	0.059
3	1.6	15	48	52	0.092
4	2.0	<6.6	10	34	0.031
5	3.2	39	35	70	0.05
6	17	11	8.9	8.4	0.11
7	79	9.9	28	4.9	0.56
8	1.2	16	8.8	9.6	0.092
9	0.79	<5.8	6.2	0.69	0.90
10	0.74	25	8.52	11	0.077
11	2.9	8.8	5.3	0.86	0.62
12	0.69	83	28	59	0.047
13	0.90	21	12	1.9	0.63
14	1.3	9.9	10	2.1	0.47
15	0.86	460	55	>570	<0.0096
16	0.56	63	62	35	0.18
17	0.14	11	11	8.4	0.13
18	2.3	99	60	170	0.035
19	0.62	75	24.4	2.7	0.91
20	>23	>230	>2,900	>580	
21	>16	>160	>2,000	>410	
22	>15	>150	>1,800	>370	

[a] The concentration required to get 50% the maximum value.
[b] The growth of murine lymphoblastoma L5178Y cells.
[c] RNA-directed DNA polymerase (AMV reverse transcriptase).
[d] The A/B value was normalized based on the concentration of diaphorase in each reaction mixture.

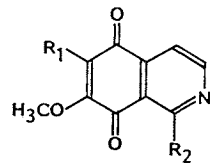
Fig. 1. Structures of heterocyclic quinones.



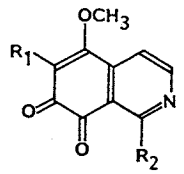
1 R_1 = H R_2 = H
2 R_1 = OCH_3 R_2 = H
3 R_1 = OCH_3 R_2 = CH_3
4 R_1 = H R_2 = OCH_3
5 R_1 = CH_3 R_2 = OCH_3



6 R = H
7 R = CH_3



8 R_1 = H R_2 = H
9 R_1 = H R_2 = CN
10 R_1 = CH_3 R_2 = H
11 R_1 = CH_3 R_2 = CN
12 R_1 = CH_3 R_2 = CH_3



13 R_1 = H R_2 = H
14 R_1 = CH_3 R_2 = H

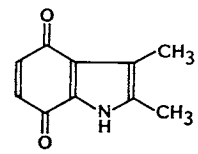


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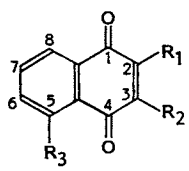
Fig. 2. Structures of naphthoquinones.



16 R_1=H R_2=H R_3=H
17 R_1=H R_2=H R_3=OH
18 R_1=CH_3 R_2=H R_3=H
19 R_1=Cl R_2=Cl R_3=H
20 R_1=OH R_2=H R_3=H
21 R_1=OH R_2=$(CH_2)_2COOH$ R_3=H
22 R_1=OH R_2=$(CH_2)_2COOC_2H_5$ R_3=H

and the single-electron reduction potential.

The inhibition of reverse transcriptase by streptonigrin or sakyomicin A was measured in the presence of NADH and bacterial diaphorase to facilitate semiquinone generation. The results shown in Fig. 3 indicate that the putative redox cycle of the antibiotics does not affect significantly the enzyme inhibitory activities of the antibiotics. According to the findings of WICK and FITZGERALD, no inhibition of reverse transcriptase was observed in the absence of dithiothreitol (DTT) in the reaction mixture, suggesting that DTT-dependent reduction of quinones might be prerequisite for the inhibition of reverse transcriptase. However, our results did not support their proposition (Fig. 4). The standard assay was conducted in the presence of 2.5 mM DTT. When the concentration of DTT was as low as 0.001 mM, the inhibition of reverse transcriptase by 3 μg/ml streptonigrin decreased to *ca.* 40% from 70~80% observed at the higher DTT concentrations (0.03~3.0 mM). However, it should be noted that the activity without any inhibitor was also depressed significantly (approximately 30% the maximum value) at 0.001 mM DTT.

Taken together, these observations lead to the conclusion that the single-electron reduction potential and the generation of semiquinone and/or oxygen radical did not determine the inhibitory activities of these antibiotics against reverse transcriptase, in contrast to the earlier postulated mechanism proposed by WICK and FITZGERALD[11] depending on one-electron reactions and generation of radicals. However, we could not exclude the possible involvement of another oxidation-reduction process in the inhibition of reverse transcriptase.

Fig. 3. Effect of *Clostridium kluyveri* diaphorase and NADH on AMV reverse transcriptase.

○ None, ● streptonigrin (3 μg/ml), △ sakyomicin A (30 μg/ml).

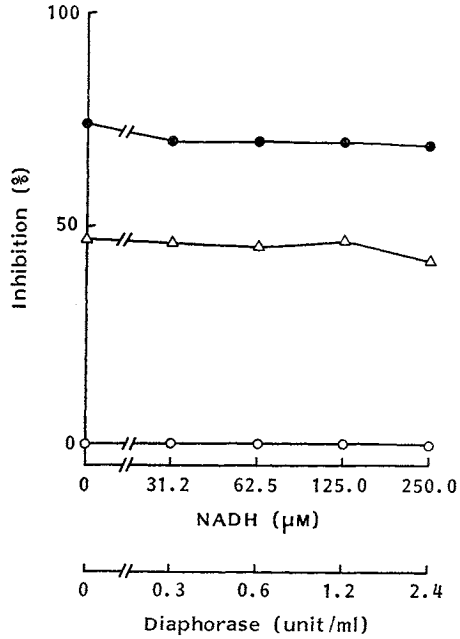
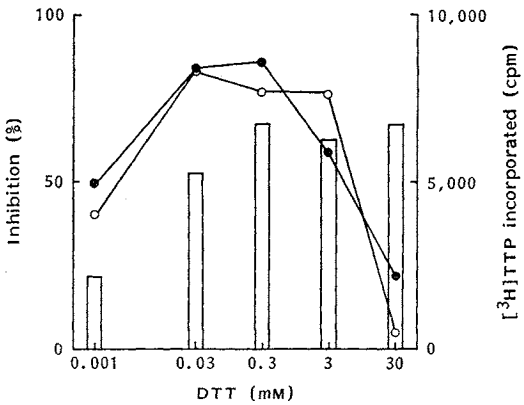


Fig. 4. Effect of DTT on AMV reverse transcriptase.

Inhibition (%): ○ Streptonigrin (3 μg/ml), ● sakyomicin A (30 μg/ml).

Open bar: The incorporation of [^{3}H]TTP into the high molecular fraction in the absence of inhibitor.



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