COMPARATIVE STUDY ON BIOLOGICAL ACTIVITIES OF HETEROCYCLIC QUINONES AND STREPTONIGRIN

YUKINORI TAKE, KEIKO OOGOSE, TAE KUBO, YOSHIO INOUYE*
and SHOSHIRO NAKAMURA

Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine,
1-2-3 Kasumi, Minami-ku, Hiroshima 734, Japan

YOSHIYASU KITAHARA and AKINORI KUBO

Meiji College of Pharmacy,
1-35-23 Nozawa, Setagaya-ku, Tokyo 154, Japan

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Thirteen heterocyclic quinones (5 quinoline quinones, 7 isoquinoline quinones, 1 indole quinone) were tested for their effects on avian myeloblastosis virus reverse transcriptase, growth of murine lymphoblastoma L5178Y cells, respiration of rat liver mitochondria and oxidation of NADH by *Clostridium kluyveri* diaphorase in comparison with those of streptonigrin, in which the quinoline quinone moiety is considered to play a crucial role.

Most of the quinoline quinones and isoquinoline quinones inhibited reverse transcriptase to the same extent as streptonigrin with the ID_{50} values ranging between 1 and 5 μg/ml, whereas the ID_{50} value of the indole quinone derivative, 4,7-dihydro-2,3-dimethylindole-4,7-dione, was 80 μg/ml. The cytotoxicities of the quinones were much lower than that of streptonigrin; the ID_{50} values of the quinones were higher than 0.15 μg/ml. In particular, the ID_{50} value of the *ortho*-quinoline quinone derivative, 8-methoxy-7-methyl-5,6-dihydroquinoline-5,6-dione, was as high as 16 μg/ml, while the 50% inhibition of cell growth was seen in the presence of 0.0025 μg/ml streptonigrin. The membrane transport of the quinones was evaluated by comparing the effects on oxygen consumption by mitochondria and oxidation of NADH by bacterial diaphorase, being proven not to be responsible for their lower cytotoxicities.

The specific inhibitors of enzymes which play a crucial role in viral replication could be sources of antiviral substances. Reverse transcriptase, required for the replication of retroviruses, will be one of the candidates for the target enzymes of antiviral substances[1,2]. In a search for the inhibitors of avian myeloblastosis virus (AMV) reverse transcriptase[3~5], streptonigrin (**1**), originally reported as an antitumor antibiotic produced by *Streptomyces flocculus*[6], was found to be one of the most potent inhibitors of this enzyme. The *in vitro* antiviral activity was also tested using an assay system consisting of human immuno-deficiency virus (HIV), a causative agent of acquired immune deficiency syndrome (AIDS) and a member of human retrovirus family[7~10], and human T-lymphotropic virus type I (HTLV-I)-carrying MT-4 cells, though the marked cytotoxicity of **1** made it difficult for its antiviral activity to be evaluated[5]. To overcome this disadvantage of **1** various amide type derivatives in the carboxyl group were synthesized[11]. Among them, the glycine derivative (**2**) seemed to be superior to **1** as an antiviral agent, since the former inhibited reverse transcriptase at the concentrations which had no effect on the growth of lymphoblastoma L5178Y cells[12,13]. The low cytotoxicity of **2** was, however, found to be attributable to its poor membrane transport[14], resulting in negligible effects on both the replication of HIV and the growth of MT-4 cells[5]. More recently, we observed that two synthetic quinoline quinone derivatives were much less toxic than **1**, while the ID_{50} values

against reverse transcriptase were comparable to that of **1**[15]. Further effort to discover specific inhibitors of reverse transcriptase has been continued employing various quinone compounds and the results concerning the biological properties of them are presented in this paper.

Materials and Methods

Materials

para-Quinoline quinones (**3**~**5**), *ortho*-quinoline quinones (**6** and **7**), *para*-isoquinoline quinones (**8**~**12**), *ortho*-isoquinoline quinones (**13** and **14**) and indole quinone (**15**) compounds were prepared as reported previously[16,17]. Diaphorase of *Clostridium kluyveri* origin was purchased from Oriental Yeast Co., Ltd., Tokyo. All the other materials were commercial products of analytical grade.

Assay Methods for Biological Activities

The details of assay methods for AMV reverse transcriptase and growth of L5178Y cells were described previously[12,18].

Determination of Hydrogen Peroxide

Hydrogen peroxide was determined by the method of TRINDER[19] with some modifications described previously[14]. Briefly, a reaction mixture (2.3 ml) consisting of 10 mM Tris-HCl (pH 8.0), 0.54 mM 4-aminoantipyrine, 0.006% phenol and 6 units/ml peroxidase was incubated with a test solution (0.2 ml) at 37°C for 20 minutes.

Respiration of Rat Liver Mitochondria

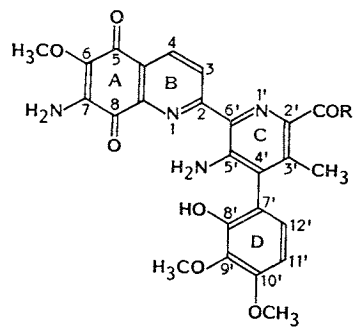
Rat liver mitochondria were isolated by the method of JOHNSON and LARDY[20] and protein concentrations were determined by the method of LOWRY *et al.*[21]. The respiration of mitochondria on glutamate was measured at 30°C by oxygen consumption using a Clark type electrode (Yellow Spring Instrument Co., Yellow Spring, Ohio). The vessel of the electrode held 3 ml of a basal medium (225 mM sucrose, 5 mM potassium phosphate and 10 mM Tris-HCl, pH 7.4) supplemented with 15 mM glutamate[14]. Mitochondria (1.5 mg as protein) and 0.37 μmol KCN were added to the electrode vessel in this order to read a rate of KCN-insensitive oxidation of glutamate by mitochondria. The test samples were dissolved in DMSO at 5 mg/ml and the final concentration of DMSO was kept at lower than 0.5%. The increase in a rate of oxygen consumption was calculated on the basis of the values before and after the addition of the test sample.

Results

The inhibition of reverse transcriptase by the quinones and **1** as a function of concentration are shown in Table 1. In general, the quinoline quinones and isoquinoline quinones were as effective as **1** as an inhibitor of AMV reverse transcriptase. The ID_{50} values of the compounds **3**, **4**, **6**~**11**, **13** and **14** were lower than 5 μg/ml, while those of **5** and **12** were 8 and 18 μg/ml, respectively. The indole quinone compound **15** was much less active than the other quinones; the ID_{50} value was *ca.* 80 μg/ml.

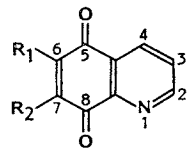
Concerning the cytotoxicity, none of the quinone compounds were comparable to **1**, as can be seen in Table 1. Especially, **6** and **7** did not cause any profound fall in the growth of

Fig. 1. Structures of streptonigrin and the glycine derivative.

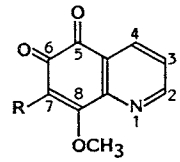


1 R=H
2 R=NHCH$_2$COOH

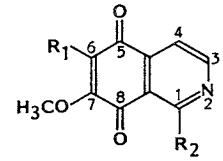
Fig. 2. Structures of the quinone compounds.



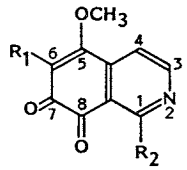
3 R_1 = H R_2 = H
4 R_1 = H R_2 = OCH_3
5 R_1 = CH_3 R_2 = OCH_3



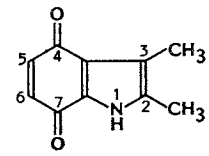
6 R = H
7 R = CH_3



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



15

Table 1. Inhibition of avian myeloblastosis virus reverse transcriptase and growth of lymphoblastoma L5178Y cells.

Compound	ID_{50} (μg/ml)	
	Reverse transcriptase	L5178Y cells
1	3	0.0025
3	2	0.32
4	<1.25[a]	0.38
5	8	0.64
6	2	3.2
7	2	16
8	3	0.22
9	<1.25[b]	0.17
10	5	0.15
11	2	0.67
12	18	0.15
13	4	0.17
14	2	0.27
15	80	0.15

[a] 64% inhibition at 1.25 μg/ml.
[b] 75% inhibition at 1.25 μg/ml.

Table 2. Effect on respiration of rat liver mitochondria.

Compound	Increased rate of O_2 consumption (μmol O_2/minute/μmol)
1	0.59
3	0.53
4	0.79
5	1.2
6	0.52
7	0.79
8	0.51
9	0.95
10	0.88
11	0.76
12	0.61
13	0.86
14	1.0
15	0.19

L5178Y cells up to 4 μg/ml.

In the presence of a catalytic amount of **1**, mitochondrial DT-diaphorase oxidizes NADH supplied by the electron transfer system with the concomitant generation of the hydroquinone of **1**, which, in turn, autoxidizes to the quinone by transferring an electron to molecular oxygen, resulting in the

Fig. 3. Generation of hydrogen peroxide coupled with the oxidation of NADH by *Clostridium kluyveri* diaphorase.

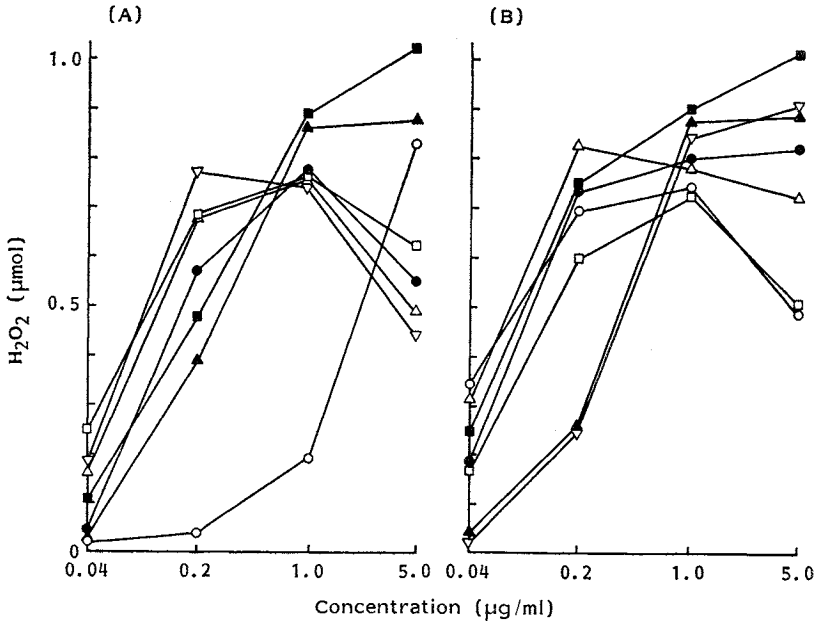
A mixture of 9.0 units/ml diaphorase, 0.9 mM NADH, 0.05 mM EDTA and 10 mM Tris-HCl (pH 8.0) was incubated in a final volume of 1 ml at 37°C for 5 minutes.

Hydrogen peroxide formed was measured by the method described in the text.

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

(A) ○ **1**, ● **3**, △ **4**, ▲ **5**, □ **6**, ■ **7**, ▽ **8**.

(B) ○ **9**, ● **10**, △ **11**, ▲ **12**, □ **13**, ■ **14**, ▽ **15**.



formation of hydrogen peroxide. The electron acceptor activity of **1** was also observed in the oxidation of NADH catalyzed by bacterial diaphorase. The former activity could be detected by measuring oxygen consumption under unaerobic conditions using oxygen electrode, and the latter by measuring hydrogen peroxide formed from molecular oxygen. As shown in Table 2 and Fig. 3, all the quinone compounds with the exception of **15** were as active as **1** in both the enzymatic and mitochondrial systems. The rate of oxygen consumption in the presence of **15** was lower than those obtained with the other quinones and **1** when compared on a molar basis. This might result from its poor membrane transport as in the case of **2**[14].

Discussion

In a previous paper[15], we reported that two quinoline quinone compounds, 6-methoxy-7-methyl-5,8-dihydroquinoline-5,8-dione and 6-methoxy-5,8-dihydroquinoline-5,8-dione, shared a potent inhibitory activity against AMV reverse transcriptase with **1**. The cytotoxicities of these two compounds (ID_{50} *ca.* 0.6 μg/ml) were, however, remarkably lower than that of **1**. Even though the quinones are superior to **1** in terms of an antiviral substance, the ID_{50} values of these compounds in cytotoxicity were still lower than the corresponding values in inhibition of reverse transcriptase. This prompted us to extend the search for the specific inhibitors of reverse transcriptase to much wide range of quinone-containing compounds.

Previously, we observed good correlativity between an electron acceptor activity in the oxidation

of NADH by *C. kluyveri* diaphorase and an inhibitory activity against reverse transcriptase using several naphthoquinone derivatives[22]. **15**, a sole indole compound among those tested in the present work, is an exception to this rule; it remains to be elucidated whether the indole structure, the side chains on C-2 and/or C-3 or the both have responsibility. We also discovered that **1** inhibited reverse transcriptase by interacting mainly with the enzyme molecule[23]. In addition to a potential to catalyze oxidation-reduction reaction, there may exist a structural requirement for the inhibitors of reverse transcriptase.

In the case of **6** and **7**, the ID_{50} values for cytotoxicity were higher than those for inhibition of reverse transcriptase; this favors their use as antiviral substances. Similar observations were made with **2**[12,13]. However, the decrease in cytotoxicity of this compound was found to result from its poor membrane transport[14]. Unlike **2**, both **6** and **7** had no defect in membrane transport as evident from the results concerning the effect on the respiration of rat liver mitochondria.

The antiviral activity of these compounds are now under investigation using *in vivo* and *in vitro* assay systems.

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References

1) MITSUYA, H.; M. POPOVIC, R. YARCHOAN, S. MATSUSHITA, R. C. GALLO & S. BRODER: Suramin protection of T cell in vitro against infectivity and cytopathic effect of HTLV-III. Science 226: 172~174, 1984
2) ROZENBAUM, W.; D. DORMONT, B. SPIER, E. VILMER, M. GINTILINI, C. GRISCELLI, L. MONTAGNIER, F. BARRÉ-SINOUSSI & J. C. CHERMANN: Antimoniotungustate (HPA 23) treatment of three patients with AIDS and one with prodome. Lancet 1985-I: 450~451, 1985
3) TANAKA, N.; T. OKABE, N. TANAKA, Y. TAKE, Y. INOUYE, S. NAKAMURA, H. NAKASHIMA & N. YAMAMOTO: Inhibition by sakyomicin A of avian myeloblastosis virus reverse transcriptase and proliferation of AIDS-associated virus (HTLV-III/LAV). Jpn. J. Cancer Res. (Gann) 77: 324~326, 1986
4) NAKASHIMA, H.; N. YAMAMOTO, Y. INOUYE & S. NAKAMURA: Inhibition by doxorubicin of human immuno-deficiency virus (HIV) infection and replication *in vitro*. J. Antibiotics 40: 396~399, 1987
5) INOUYE, Y.; Y. TAKE, S. NAKAMURA, H. NAKASHIMA, N. YAMAMOTO & H. KAWAGUCHI: Screening for inhibitors of avian myeloblastosis virus reverse transcriptase and effect on the replication of AIDS-virus. J. Antibiotics 40: 100~104, 1987
6) RAO, K. V. & W. P. CULLEN: Streptonigrin, an antitumor substance. I. Isolation and characterization. *In* Antibiotics Annual 1959~1960. *Eds.*, H. WELCH & F. MARTI-IBAÑEZ, pp. 950~953, Medical Encyclopedia Inc., New York, 1960
7) BARRÉ-SINOUSSI, F.; J. C. CHERMANN, F. REY, M. T. NUGEYRE, S. CHAMARET, J. GRUEST, C. DAUGUET, C. AXLER-BLIN, F. VÉZINET-BRUN, C. ROUZIOUX, W. ROZENBAUM & L. MONTAGNIER: Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science 220: 868~871, 1983
8) BRODER, S. & R. C. GALLO: A pathogenic retrovirus (HTLV-III) linked to AIDS. N. Engl. J. Med. 311: 1292~1297, 1984
9) KLATZMANN, D.; F. BARRÉ-SINOUSSI, M. T. NUGEYRE, C. DAUGUET, E. VILMER, C. GRISCELLI, F. BRUN-VEZINET, C. ROUZIOUX, J. C. GLUCKMAN, J.-C. CHERMANN & L. MONTAGNIER: Selective tropism of lymphadenopathy associated virus (LAV) for helper-inducer T lymphocytes. Science 225: 59~63, 1984
10) POPOVIC, M.; M. G. SARNGADHARAN, E. READ & R. C. GALLO: Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. Science 224: 497~500, 1984
11) MIYASAKA, T.; S. HIBINO, Y. INOUYE & S. NAKAMURA: Synthesis of novel streptonigrin-2-amide derivatives with 3,3'-(phenylphosphonyl)bis(1,3-thiazolidine-2-thione). J. Chem. Soc. Perkin Trans. I 1986: 479~482, 1986
12) INOUYE, Y.; H. OKADA, S. K. ROY, T. MIYASAKA, S. HIBINO, N. TANAKA & S. NAKAMURA: Biological properties of streptonigrin derivatives. I. Antimicrobial and cytocidal activities. J. Antibiotics 38: 1429~1432, 1985

13) OKADA, H.; H. MUKAI, Y. INOUYE & S. NAKAMURA: Biological properties of streptonigrin derivatives. II. Inhibition of reverse transcriptase activity. J. Antibiotics 39: 306~308, 1986
14) INOUYE, Y.; H. OKADA, J. UNO, T. ARAI & S. NAKAMURA: Effects of streptonigrin derivatives and sakyomicin A on the respiration of isolated rat liver mitochondria. J. Antibiotics 39: 550~556, 1986
15) INOUYE, Y.; Y. TAKE, K. OOGOSE, A. KUBO & S. NAKAMURA: The quinoline quinone as the minimum entity for reverse transcriptase inhibitory activity of streptonigrin. J. Antibiotics 40: 105~107, 1987
16) KITAHARA, Y.; S. NAKAHARA, R. NUMATA, K. INABA & A. KUBO: The assignment of the carbon-13 nuclear magnetic resonance spectra of isoquinoline and quinoline quinones. Chem. Pharm. Bull. 33: 823~830, 1985
17) KITAHARA, Y.; S. NAKAHARA, R. NUMATA & A. KUBO: Synthesis of 4,7-indolequinones. The oxidative demethylation of 4,7-dimethoxyindoles with ceric ammonium nitrate. Chem. Pharm. Bull. 33: 2122~2128, 1985
18) HANAJIMA, S.; K. ISHIMARU, K. SAKANO, S. K. ROY, Y. INOUYE & S. NAKAMURA: Inhibition of reverse transcriptase by limocrocin. J. Antibiotics 38: 803~805, 1985
19) TRINDER, P.: Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor. Ann. Clin. Biochem. 6: 24~27, 1969
20) JOHNSON, D. & H. LARDY: Isolation of liver or kidney mitochondria. Methods Enzymol. 10: 94~96, 1967
21) LOWRY, O. H.; N. J. ROSEBROUGH, A. L. FARR & R. J. RANDALL: Protein measurement with the Folin phenol reagent. J. Biol. Chem. 19: 265~275, 1951
22) TAKE, Y.; M. SAWADA, H. KUNAI, Y. INOUYE & S. NAKAMURA: Role of the naphthoquinone moiety in the biological activities of sakyomicin A. J. Antibiotics 39: 557~563, 1986
23) OKADA, H.; Y. INOUYE & S. NAKAMURA: Kinetic analysis of inhibition of reverse transcriptase by streptonigrin. J. Antibiotics 40: 230~232, 1987