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INHIBITION OF HIV-REVERSE TRANSCRIPTASE ACTIVITY BY ASTERRIQUINONE AND ITS ANALOGUES

Katsuhiko ONO,[1] Hideo NAKANE,[1] Sakae SHIMIZU,[2] and Saburo KOSHIMURA[3]

[1]Laboratory of Viral Oncology, Aichi Cancer Center Research Institute, Chikusa-ku, Nagoya 464, Japan

[2]Department of Biochemistry, Faculty of Pharmaceutical Sciences, Kanazawa University, Takara-machi 13-1, Kanazawa 920, Japan

[3]Department of Serology, Kanazawa Medical University, Uchinada-machi 1-1, Kahoku-gun, Ishikawa 920-02, Japan

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SUMMARY: Asterriquinone (ARQ; 2,5-bis-[1'-(1",1"-dimethyl-2"-propenyl)-indol-3'-yl]-3,6-dihydroxy-1,4-benzoquinone) and its three analogues [*i.e.*, 3,6-dihydroxy-2-[2'-(1",1"-dimethyl-2"-propenyl)-indol-3'-yl]-5-[1',7'-(1",1"-dimethylpropano)-indol-3'-yl]-1,4-benzoquinone (B1-4), 3,6-dihydroxy-2-[2'-(1",1"-dimethyl-2"-propenyl)-indol-3'-yl]-5-indol-3'-yl-1,4-benzoquinone (C1-1) and 3,6-dihydroxy-2,5-diindol-3'-yl-1,4-benzoquinone (D-1)] were found to be strong inhibitors of the activity of reverse transcriptase from human immunodeficiency virus type-1. Under the reaction conditions employed, the enzyme activity was inhibited by more than 70% in the presence of 10 μM each of these compounds. The mode of inhibition by these compounds was competitive with respect to the template·primer, $(rA)_n \cdot (dT)_{12-18}$, and noncompetitive with respect to the triphosphate substrate, dTTP. The Ki values of HIV-1 reverse transcriptase were determined to be 2.3, 1.5, 0.1 and 0.3 μM for ARQ, B1-4, C1-1 and D-1, respectively.

In search for effective antiretroviral agents, virus-associated reverse transcriptase has been regarded as an appropriate target, since the step of reverse transcription from viral RNA to proviral DNA is unique for retrovirus infection to and multiplication in the host cells. In fact, many reverse transcriptase inhibitors have been proved inhibitory to human immunodeficiency virus (HIV) replication *in vitro* and/or *in vivo* (1).

Abbreviations: HIV, human immunodeficiency virus; suramin, hexasodium *sym*-bis(*m*-amino-*p*-methylbenzoyl-1-naphthylamino-4,6,8-trisulfonate)carbamide; HPA23, heteropolyanion 23 (21-tungsto-9-antimonate ammonium salt); AZT, 3'-azido-3'-deoxythymidine; DDC, 2',3'-dideoxycytidine; DDI, 2',3'-dideoxyinosine; AIDS, acquired immune deficiency syndrome; ARQ (asterriquinone), 2,5-bis-[1'-(1",1"-dimethyl-2"-propenyl)-indol-3'-yl]-3,6-dihydroxy-1,4-benzoquinone; B1-4, 3,6-dihydroxy-2-[2'-(1",1"-dimethyl-2"-propenyl)-indol-3'-yl]-5-[1',7'-(1",1"-dimethylpropano)-indol-3'-yl]-1,4-benzoquinone; C1-1, 3,6-dihydroxy-2-[2'-(1",1"-dimethyl-2"-propenyl)-indol-3'-yl]-5-indol-3'-yl-1,4-benzoquinone; D-1, 3,6-dihydroxy-2,5-diindol-3'-yl-1,4-benzoquinone.

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These include, for example, suramin (2,3), 21-tungsto-9-antimonate ammonium salt (HPA23) (4), 3'-azido-3'-deoxythymidine (AZT) (5,6), 2',3'-dideoxycytidine (DDC) (7,8), 2',3'-dideoxyinosine (DDI) (7) and various other 2',3'-dideoxynucleosides (7). When these chemically synthesized compounds were given to the patients with acquired immune deficiency syndrome (AIDS) or AIDS-related complex, however, various side effects appeared during the course of clinical trials; *e.g.*, renal insufficiency for suramin, thrombocytopenia for HPA23 (4), anemia and leucopenia for AZT (9), and peripheral neuropathy for DDC (10) and DDI, *etc.* Both clinical and/or immunological improvements have thus been obtained at the expense of serious secondary effects listed above. Such side effects of the compounds seem to be due, at least a part, to their inhibitory effects on cellular DNA polymerases responsible for host cell DNA replication and/or repair (1).

Besides the chemically synthesized compounds, various natural products and the components isolated thereof have been shown to be inhibitory to the activity of HIV-reverse transcriptase. Chinese and Japanese herbal extracts (11), a Kampo drug, Sho-Saiko-To (12) and various flavonoids (13,14) are typical examples, and some of them are considered to be candidates for potential antiretroviral agents. We describe in this paper that some fungi-originated asterriquinone (ARQ) and some of its analogues are also strong inhibitors of HIV-reverse transcriptase.

MATERIALS AND METHODS

Chemicals. ARQ and its analogues were prepared from *Aspergillus terreus*, strains IFO 6123 and 8835 as described previously (15-17). Structural formulae of asterriquinone and its analogues examined in this study are shown in Fig. 1. The sources of other materials used in this work were as follows:

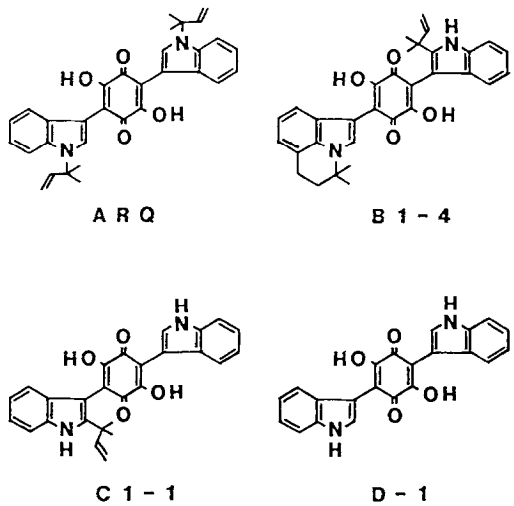


Fig. 1. Structural formulae of asterriquinone and its analogues examined in this study.

[^{3}H]dTTP from Amersham International (Amersham, England); unlabeled dTTP, poly(rA), oligo(dT) from P-L Biochemicals, Inc. (Milwaukee, WI); and DEAE-cellulose paper disc (DE81, diameter 23 mm) from Whatman Ltd. (Springfield Mill, Maidstone, Kent, England).

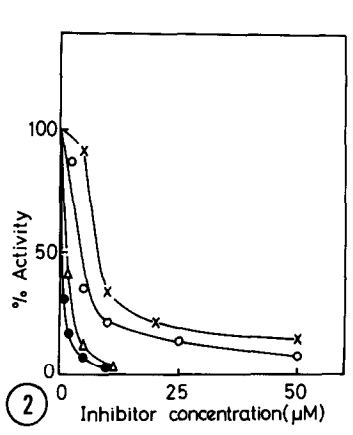
HIV-reverse transcriptase. HIV-1 reverse transcriptase was purified from *E. coli* harboring an expression plasmid for the precise coding sequence of the enzyme. The purified enzyme was a generous gift from Dr. S.H. Wilson, NIH, USA.

Assay for reverse transcriptase. Reverse transcriptase activity was measured with $(rA)_n \cdot (dT)_{12-18}$ as the template·primer under the optimized reaction conditions for HIV-1 reverse transcriptase. The reaction mixture contained the following components: 50 mM Tris-HCl, pH 8.0; 4 μg/ml $(rA)_n \cdot (dT)_{12-18}$ (base ratio, 1:1); 10 μM [^{3}H]dTTP (400 cpm/pmole); 5 mM dithiothreitol; 50 mM KCl; 15% (v/v) glycerol; and 5 mM $MgCl_2$. All incubations (50 μl) were carried out at 37°C for 30 min, and the reaction was stopped by adding 20 μl of 0.2 M EDTA and immersing the mixture in ice. Then, 50 μl of the mixture was transferred to a DE81 filter paper disc and processed for radioactivity counting as previously described (18).

RESULTS

Inhibition of HIV-1 reverse transcriptase by asterriquinone and its analogues.

The effects of asterriquinone and its analogues on the activity of HIV-1 reverse transcriptase were examined under the reaction conditions described in Materials and Methods. As shown in Fig. 2, the enzyme activity was



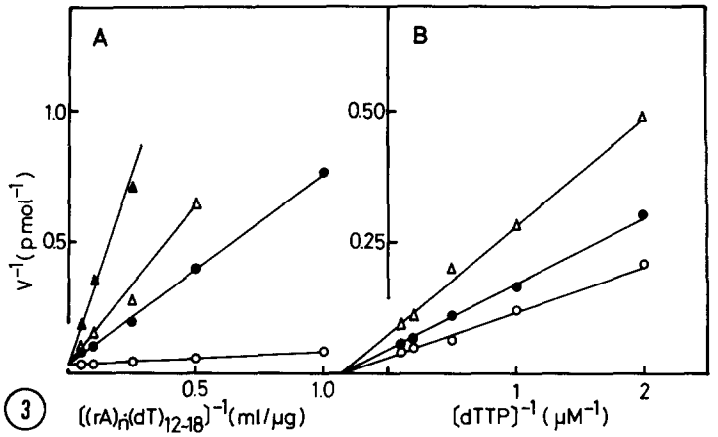


Fig. 2. Effects of asterriquinone and its analogues on the activity of HIV-1 reverse transcriptase. Reverse transcriptase activity was measured under the conditions described in Materials and Methods in the presence of various concentrations of asterriquinone and its analogues as indicated in the figure, by determining the incorporation of [^{3}H]dTMP with $(rA)_n \cdot (dT)_{12-18}$ as the template·primer. The compounds tested and the symbols used are; asterriquinone (x), B1-4 (o), C1-1 (●), and D-1 (△). The 100% value was 94.4 pmoles.

Fig. 3. Analysis of the inhibition of HIV-1 reverse transcriptase activity by asterriquinone analogues. Reactions were carried out under the conditions described in Materials and Methods, except that various concentrations of $(rA)_n \cdot (dT)_{12-18}$ (A) and [^{3}H]dTTP (B) were used as the template·primer and the triphosphate substrate, respectively, in the presence of various concentrations of D-1 (A) and B1-4 (B), respectively. D-1 concentrations in Panel A were 0 (o), 1.5 (●), 2.0 (△), and 3.0 (▲) μM, and B1-4 concentrations in Panel B were 0 (o), 5 (●), and 8 (△) μM.

inhibited by approximately 90% in the presence of 5 μM C1-1 or D-1 and by 75% in the presence of the same concentration of B1-4. The degree of inhibition was dose-dependent and nearly 100% inhibition was observed at 10 μM C1-1 or D-1. The original compound asterriquinone was, however, less effective in inhibition of HIV-1 reverse transcriptase activity than were its three analogues (Fig. 2).

Analysis of the mode of inhibition and determination of inhibition constants.
The mode of inhibition of reverse transcriptase activity was analyzed by changing the concentrations of either the template·primer or the triphosphate substrate in the presence of various concentrations of each of the inhibitors. Any of these asterriquinone and its analogues inhibited the enzyme activity competitively with respect to the template·primer, $(rA)_n \cdot (dT)_{12-18}$ and noncompetitively with respect to the triphosphate substrate, dTTP. Typical examples are shown in Fig. 3A and B for D-1 and B1-4, respectively. The Ki values for D-1 was determined to be 0.3 μM by replotting (Dixon plot) the data in Fig. 3A, and was summarized with other Ki values for ARQ, B1-4 and C1-1 in Table 1.

Enzyme specificity of the inhibition by asterriquinone and its analogues.
To know the enzyme specificity of the inhibition, effects of asterriquinone and its analogues on the activities of DNA polymerases α, β and γ purified from KBIII cells were examined under the reaction conditions described previously (14). The results are expressed as IC_{50} values (inhibitor concentrations at which 50% inhibition of the enzyme activities were achieved) and are summarized in Table 2. As seen in this table, any of B1-4, C1-1 and D-1 is more or less inhibitory to cellular DNA polymerases than to HIV-reverse transcriptase. DNA polymerase α is particularly sensitive to inhibition by C1-1 and D-1 as shown by low IC_{50} values (1.4 and 0.8 μM, respectively).

Table 1. Characterization of inhibition of HIV-reverse transcriptase by asterriquinone and its analogues

Variable substrate	Km[a]	Asterriquinone and its analogues							
		ARQ		B1-4		C1-1		D-1	
		Mode	Ki(μM)	Mode	Ki(μM)	Mode	Ki(μM)	Mode	Ki(μM)
$(rA)_n \cdot (dT)_{12-18}$(1:1)	0.76	C[b]	2.3	C	1.5	C	0.1	C	0.3
dTTP	4.4	NC[b]		NC		NC		NC	

[a]Km values are expressed in μg/ml and μM for the template and the triphosphate substrate respectively.

[b]C, competitive; NC, noncompetitive.

Table 2. IC_{50} values of asterriquinone and its analogues in the inhibition of various DNA polymerases[a]

DNA polymerase	Template·primer and concentration (μg/ml)	Asterriquinone and its analogues (IC_{50})			
		ARQ	B1-4	C1-1	D-1
		μM	μM	μM	μM
Cellular:					
α	Activated DNA, 80	NI[c]	22	1.4	0.8
β	$(rA)_n \cdot (dT)_{12-18}$(1:2)[b],30	20	9	2.9	4.1
γ	$(rA)_n \cdot (dT)_{12-18}$(10:1),11	NI	16	3.1	2.2
HIV-reverse transcriptase	$(rA)_n \cdot (dT)_{12-18}$(1:1),4	8.5	4.5	0.9	1.8

[a]Assay conditions are described elsewhere (14).

[b]Numbers in parentheses are base ratios of the template to the primer.

[c]NI, no inhibition.

DISCUSSION

ARQ and some of its analogues originated from fungi were previously shown to have inhibitory effects on the growth of some transplantable animal tumors, Ehrlich carcinoma, ascites hepatoma AH13 and mouse P388 leukemia (19,20). This ARQ and its analogues were found to be strong inhibitors of various DNA polymerases, indicating that the inhibitory effects of these compounds on cellular DNA synthesis is a possible mechanism of their anti-tumor activity (K. Ono, *et al.*, manuscript in preparation). Extensive studies revealed that ARQ and its three analogues tested in the present study were also potent inhibitors for HIV-reverse transcriptase (Fig. 2). Of the four compounds tested, inhibition potentials of C1-1 and D-1 were particularly strong as shown by their low Ki values (0.1 and 0.3 μM, respectively) (Table 1). Kinetic analysis revealed that the inhibition by these compounds was due to competition between the inhibitor and the template·primer, $(rA)_n \cdot (dT)_{12-18}$ (Table 1; Fig. 3). Unfortunately, however, the highly purified preparation of HIV-reverse transcriptase used in this study was unable to utilize another interesting template·primer, $(rC)_n \cdot (dG)_{12-18}$, and, therefore, it was impossible to examine whether these ARQ analogues compete with this pyrimidine template.

The previous experiments carried out to clarify structure-activity relationship of ARQ analogues revealed that the presence of free hydroxyl groups in the benzoquinone moiety and of tert- or iso-pentenyl groups in the indol ring of ARQ is necessary to exhibit the antitumor activity (21). Two free hydroxyl groups at positions 3 and 6 of benzoquinone moiety are also indispensable for the inhibition of the activities of DNA polymerases and

HIV-reverse transcriptase, since methylation or acetylation of these hydroxyl groups resulted in complete loss of the inhibitory activities of ARQ and its analogues (Ono, *et al.*, unpublished observation). Difference in inhibition potential among the four compounds tested seems, therefore, to be due to the presence (ARQ, B1-4 and C1-1) or absence (D-1) of the different groups introduced on indol ring of these compounds (Fig. 1).

HIV-reverse transcriptase has stronger affinities to C1-1 and D-1 than to ARQ and B1-4, as shown by their Ki values (Table 1). Both C1-1 and D-1 are, however, strongly inhibitory also to cellular DNA polymerases α, β and γ (Table 2). Whether or not ARQ and its analogues are effective in inhibition of HIV replication depends on the forthcoming results of anti-HIV and toxicity tests in intact cell culture system now in progress.

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