

INHIBITION OF REVERSE TRANSCRIPTASE ACTIVITY BY A FLAVONOID
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SUMMARY: 5,6,7-Trihydroxyflavone (baicalein) is a potent inhibitor of the activities of reverse transcriptases from murine leukemia viruses (MLV) (Rauscher and Moloney strains) and human immunodeficiency virus (HIV). Under the reaction conditions specified for each of the MLV- and HIV-reverse transcriptases, both enzyme activities were inhibited by more than 90% in the presence of 2 µg/ml baicalein. The mode of the inhibition by baicalein was competitive with respect to the template·primer, (rA)_n·(dT)₁₂₋₁₈, and noncompetitive to dTTP substrate. K_i value of baicalein for the MLV-reverse transcriptase was determined to be 0.37 µM. © 1989 Academic Press, Inc.

Some antiretroviral compounds have now attracted special attention in the strategies for chemotherapy of a retrovirus-associated human disease (AIDS) and most of the antiretroviral compounds so far proven effective appear to be targeted at the virus-associated reverse transcriptase. In fact, a number of chemically synthesized inhibitors of reverse transcriptase have been described to inhibit HIV replication *in vitro* or *in vivo*: i.e., suramin (1), HPA23 (2), 3'-azido-2',3'-dideoxythymidine (AZT) (3,4) and various 2',3'-dideoxynucleosides (5,6). Unfortunately, however, the clinical trials for the administration of these compounds to AIDS patients revealed serious side effects such as thrombocytopenia for HPA23 (2),

Abbreviations: baicalein, 5,6,7-trihydroxyflavone; AIDS, acquired immune deficiency syndrome; HIV, human immunodeficiency virus; suramin, hexasodium *sym*-bis(*m*-aminobenzoyl-*m*-amino-*p*-methylbenzoyl-1-naphthylamino-4,6,8-trisulfonate)carbamide; HPA23, heteropolyanion 23(21-tungsto-9-antimoniate ammonium salt); AZT, 3'-azido-3'-deoxy-thymidine; MLV, murine leukemia virus.

anemia and leucopenia for AZT (7), etc., and at least a part of these side effects seems to be due to the inhibitory effects of the antiretroviral compounds on cellular DNA polymerases (8,9). It seems that a better strategy to find novel antivirals (inhibitors of the reverse transcriptase) with less cytotoxicity is to look for natural substance(s) in our surroundings. Flavonoids, for example, are known to be popular compounds widely distributed among various plants. During the course of our screening test with various flavonoids, we found that 5,6,7-trihydroxyflavone strongly inhibited the activities of MLV- and HIV-reverse transcriptases. This paper describes some of the details of this finding.

MATERIALS AND METHODS

Chemicals. 5,6,7-Trihydroxyflavone (trivial name: baicalein (Fig. 1) was the product of Tsumura & Co., Tokyo, Japan. The sources of other materials used in this work were as follows: [³H]dTTP from Amersham International (Amersham, England); unlabeled dTTP, poly(rA), oligo(dT) from P-L Biochemicals, Inc. (Milwaukee, WI, USA); activated calf thymus DNA from Worthington Biochem. Corp. (Freehold, N.J., USA); and DEAE-cellulose paper disc (DE81, diameter 23 mm) from Whatman Ltd. (Springfield Mill, Maidstone, Kent, England).

Reverse transcriptases. Rauscher murine leukemia virus (R-MLV) was obtained from the culture medium of an established virus-producing cell line, R-17, and reverse transcriptase was purified on a DEAE-Sephadex A-50 column as previously described (10). Moloney murine leukemia virus (Mo-MLV) was collected from the culture medium and purified by ultracentrifugation in a sucrose gradient as described earlier (11). Detergent-treated (0.1% Triton X-100) Mo-MLV particles were used as Mo-MLV-reverse transcriptase. HIV1-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.

DNA polymerases. DNA polymerases α , β and γ were purified from KBIII cells, as previously described for DNA polymerase α (12), β (13) and γ (14) with some modifications.

Assay for reverse transcriptase and DNA polymerase activities. Reverse transcriptase activity was measured with (rA)_n·(dT)₁₂₋₁₈ as the template-primer under the optimized reaction conditions specified for each of the MLV- and HIV-reverse transcriptases (8). The reaction mixture contained the following components: 50 mM Tris-HCl, pH 8.0; 20 μ g/ml (rA)_n·(dT)₁₂₋₁₈ (1:1); 10 μ M [³H]dTTP (400 cpm/pmole); 5 mM dithiothreitol; 50 mM KCl; 15% (v/v) glycerol; 0.2 mM MnCl₂ for MLV- and HIV-reverse transcriptase and 5 mM MgCl₂ for HIV-reverse transcriptase.

The reaction mixtures to measure the other DNA polymerase activities contained the following components. For DNA polymerase α ; 50 mM Tris-HCl

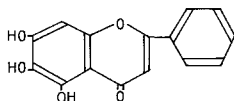


Fig. 1: Structural formula of 5,6,7-trihydroxyflavone (baicalein).

(pH 7.5), 80 $\mu\text{g/ml}$ activated calf thymus DNA, 10 μM each of dATP, dCTP, dGTP and [^3H]dTTP (1000 cpm/pmole), 5 mM dithiothreitol, 15% (v/v) glycerol, and 4 mM MgCl_2 . For DNA polymerases β ; 50 mM Tris-HCl (pH 8.0), 30 $\mu\text{g/ml}$ $(\text{rA})_n \cdot (\text{dT})_{12-18}$ (1:2), 10 μM [^3H]dTTP (400 cpm/pmole), 5 mM dithiothreitol, 100 mM KCl, 15% (v/v) glycerol, and 0.2 mM MnCl_2 . For DNA polymerase γ ; 50 mM Tris-HCl (pH 7.5), 11 $\mu\text{g/ml}$ $(\text{rA})_n \cdot (\text{dT})_{12-18}$ (10:1), 1 μM [^3H]dTTP (6000 cpm/pmole), 5 mM dithiothreitol, 70 mM KCl, 15% (v/v) glycerol, and 0.1 mM MnCl_2 .

The reaction (50 μl total volume) was started by adding 5 μl enzyme, and the reaction mixture was incubated at 37°C for 30 min and stopped by adding 20 μl 0.2 M EDTA and immersion in ice. Then a 50- μl aliquot of the mixture was transferred to a DE81 filter paper disc and processed for radioactivity counting as described by Lindell *et al.* (15).

RESULTS

Inhibition of various reverse transcriptase activities by 5,6,7-trihydroxyflavone (baicalein). The effects of baicalein on the activities of various reverse transcriptases were examined under the assay conditions described in Materials and Methods. As shown in Fig. 2, the activities of MLV-reverse transcriptases were inhibited by approximately 90% in the presence of 1 $\mu\text{g/ml}$ baicalein and the activity of HIV-reverse transcriptase was inhibited by 90% in the presence of 2 $\mu\text{g/ml}$ baicalein.

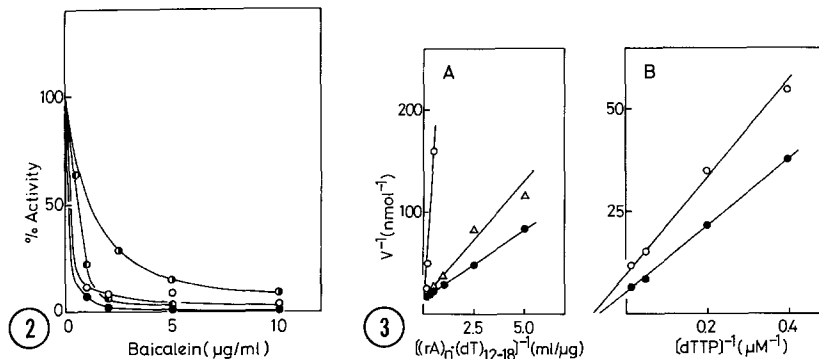


Fig. 2: Effect of baicalein on the activities of MLV- and HIV-reverse transcriptases. Reverse transcriptase activity was measured under the conditions described in Materials and Methods in the presence of various concentrations of baicalein as indicated in the figure, by determining the incorporation of [^3H]dTMP with $(\text{rA})_n \cdot (\text{dT})_{12-18}$ as the template-primer. The reverse transcriptases tested were from; R-MLV (\bullet), Mo-MLV (\circ), and HIV (\bullet , \circ). HIV-reverse transcriptase activity was measured in the presence of 5 mM Mg^{2+} (\circ) and 0.2 mM Mn^{2+} (\bullet). 100% values (pmole) were 6.8 (\bullet), 4.0 (\circ), 94.4 (\circ), and 5.0 (\bullet).

Fig. 3: Analysis of the inhibition of R-MLV-reverse transcriptase by baicalein. Reactions were carried out under the conditions described in Materials and Methods, except that various concentrations of $(\text{rA})_n \cdot (\text{dT})_{12-18}$ (A) and [^3H]dTTP (B) were used as the template-primer and the triphosphate substrate, respectively, in the presence of various concentrations of baicalein. Baicalein concentrations were; 0 (\bullet), 0.1 (Δ), and 0.3 (\circ) $\mu\text{g/ml}$. The figure represents double-reciprocal plots.

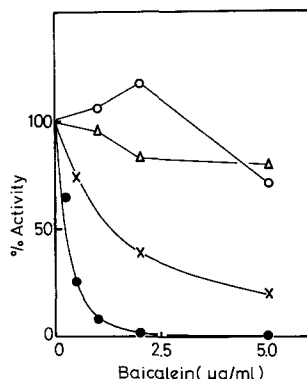


Fig. 4: Effect of baicalein on the activities of DNA polymerases α , β , and γ . DNA polymerase activities were measured by determining the incorporation of [^3H]dTMP under the conditions described in Materials and Methods in the presence of various concentrations of baicalein as indicated in the figure. DNA polymerase α (o), β (Δ) and γ (x), and R-MLV-reverse transcriptase (\bullet). 100% values (pmole) were 14.0 (o), 7.5 (Δ), 0.29 (x), and 13.2 (\bullet).

Analysis of mode of inhibition and determination of the inhibition constant.

The inhibition of the reverse transcriptase activity by baicalein was analyzed with R-MLV-reverse transcriptase by changing the concentrations of either the template·primer or the triphosphate substrate in the presence of various concentrations of baicalein. As shown in the double-reciprocal plots in Fig. 3, baicalein inhibited the activity of R-MLV-reverse transcriptase competitively with respect to the template·primer, $(\text{rA})_n \cdot (\text{dT})_{12-18}$ and noncompetitively with respect to the triphosphate substrate, dTTP. This indicates that baicalein binds to the binding-site of the template·primer on the enzyme and, consequently, interfere with the reaction. K_i value of the R-MLV-reverse transcriptase for baicalein was determined by replotting (Dixon plot) the data in Fig. 3A to be 0.37 μM .

Enzyme specificity of the inhibition by baicalein. To evaluate the enzyme specificity of the inhibition, the effects of baicalein on the activities of DNA polymerases α , β and γ were examined under the assay conditions described in Materials and Methods. As shown in Fig. 4, DNA polymerases α and β were virtually insensitive to inhibition by baicalein at concentrations up to 5 $\mu\text{g/ml}$, whereas DNA polymerase γ was moderately inhibited by this compound. However, the degree of the inhibition of γ -polymerase was much less than that of R-MLV-reverse transcriptase.

DISCUSSION

Only a few natural ingredients of plants have been reported so far to be inhibitory to reverse transcriptase and cellular DNA polymerases. Some tannins, for example, inhibit reverse transcriptase from avian myeloblastosis virus (16), and gossypol, isolated from cotton seed, inhibits

DNA polymerase α from HeLa cells (17). In order to find more selective inhibitors for reverse transcriptase, we have screened some flavonoids from various plants.

Baicalein was the first one which was found to be a potent inhibitor of the activities of reverse transcriptases from MLV and HIV, while it was not inhibitory to those of DNA polymerases α and β . Only DNA polymerase γ was moderately sensitive to baicalein. Thus baicalein seems to be highly specific for reverse transcriptase. It inhibited the R-MLV-reverse transcriptase activity in competitive fashion with respect to the template-primer, $(rA)_n \cdot (dT)_{12-18}$, with the K_i value of $0.37 \mu\text{M}$. HIV-reverse transcriptase activity has usually been assayed in the presence of Mg^{2+} as the divalent cation (18). However, the degree of the inhibition of HIV-reverse transcriptase was much higher when the enzyme activity was measured in the presence of Mn^{2+} than in the presence of Mg^{2+} (Fig. 2), indicating stronger affinity of baicalein to the enzyme molecule with Mn^{2+} as the divalent cation.

Flavonoids are widely distributed among plants and there are various flavonoid compounds the structures of which resemble baicalein. We are evaluating the inhibitory effects of these compounds on the reverse transcriptase activity in comparison with those on cellular DNA polymerases. Such comparative study will provide useful informations as to the "structure-activity relationship" among this class of the compounds.

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