Volume 286, number 1,2, 83–85 FEBS 09920 July 1991

ADONIS 0014579391006280

Inhibition of HIV-reverse transcriptase activity by some phloroglucinol derivatives

Hideo Nakane[1], Munehisa Arisawa[2], Akio Fujita[2], Saburo Koshimura[3] and Katsuhiko Ono[1]

[1] *Laboratory of Viral Oncology, Aichi Cancer Center Research Institute, Chikusa-ku, Nagoya 464,* [2] *Department of Medical Resources, Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-01 and* [3] *Department of Serology, Kanazawa Medical University, 1-1 Daigaku, Uchinadamachi, Kahokugun, Ishikawa 920-02, Japan*

Received 23 April 1991

Four phloroglucinol derivatives, named mallotophenone (5-methylene-*bis*-2,6-dihydroxy-3-methyl-4-methoxyacetophenone), mallotochromene (8-acetyl-5,7-dihydroxy-6-(3-acetyl-2,4-dihydroxy-5-methyl-6-methoxybenzyl)-2,2-dimethylchromene), mallotojaponin (3-(3,3(dimethylallyl)5-(3(acetyl-2,4-dihydroxy-5-methyl-6-methoxybenzyl)-phloracetophenone) and mallotolerin (3-(3-methyl-2-hydroxybut-3-enyl)-5-(3-acetyl-2,4-dihydroxy-5-methyl-6-methoxybenzyl)-phloracetophenone), have been tested for their ability to inhibit the activity of human immunodeficiency virus (HIV)-reverse transcriptase. Under the reaction conditions with $(rA)_n \cdot (dT)_{12-18}$ as the template·primer, the enzyme activity was inhibited by approximately 70% in the presence of 10 μg/ml mallotochromene or mallotojaponin, whereas mallotophenone and mallotolerin were much less inhibitory to the enzyme. The enzyme activity was also inhibited, though to lesser extent, by these compounds under similar conditions with initiated MS-2 phage RNA as the template·primer. The mode of inhibition was, as analyzed with mallotojaponin, competivite with respect to the template·primer, $(rA)_n \cdot (dT)_{12-18}$, and non-competitive with respect to the triphosphate substrate, dTTP. The K_i value of mallotojaponin for HIV-reverse transcriptase was determined to be 6.1 μM.

Phloroglucinol derivative; Anti-reverse transcriptase activity; Inhibition mechanism; Acquired immunodeficiency syndrome

1. INTRODUCTION

The recent search for new antiretrovirals for treatment of acquired immunodeficiency syndrome (AIDS) has been directed mainly to well-characterized compounds such as nucleoside analogues with an expected mode of action. These chemically synthesized compounds have, however, revealed various side effects when administered to patients with AIDS or AIDS-related complex (ARC); e.g. anemia and leucopenia for 3′-azido-3′-deoxythymidine (AZT) [1] and peripheral neuropathy for 2′,3′-dideoxycytidine (DDC) [2] and 2′,3′-dideoxyinosine (DDI) [3,4].

Some traditional medicines, mainly composed of plant extracts, have therefore attracted special attention, since they are known to be non-toxic by a long practical experience [5] and they may contain yet unknown new compounds with antiviral activity. In search for inhibitor(s) of human immunodeficiency virus (HIV)-reverse transcriptase, it was found that a Kampo drug, Sho-saiko-to, was inhibitory not only to this enzyme activity [6], but also to HIV replication in lymphocyte cultures of HIV-seropositive asymptomatic and ARC subjects [7]. An active component of Sho-saiko-to has been identified as 5,6,7-trihydroxyflavone (baicalein) which strongly inhibits HIV-reverse transcriptase activity [8]. Besides baicalein, some other flavonoids were shown to be potent inhibitors of HIV-reverse transcriptase [9].

In an extensive survey of plant extracts, we found that two new phloroglucinol derivatives, mallotojaponin and mallotochromene, isolated from the pericarps of *Mallotus japonicus* had strong anti-reverse transcriptase activity. This paper describes this finding together with their action mechanism.

Abbreviations: AIDS, acquired immunodeficiency syndrome; ARC, AIDS-related complex; HIV, human immunodeficiency virus; baicalein, 5,6,7-trihydroxyflavone; mallotophenone, 5-methylene-*bis*-2,6-dihydroxy-3-methyl-4-methoxyacetophenone; mallotochromene, 8-acetyl-5,7-dihydroxy-6-(3-acetyl-2,4-dihydroxy-5-methyl-6-methoxybenzyl)-2,2-dimethylchromene; mallotojaponin, 3-(3,3-dimethylallyl)-5-(3-acetyl-2,4-dihydroxy-5-methyl-6-methoxybenzyl)-phloracetophenone; mallotolerin, 3-(3-methyl-2-hydroxybut-3-enyl)-5-(3-acetyl-2,4-dihydroxy-5-methyl-6-methoxybenzyl)-phloracetophenone

Correspondence address: K. Ono, Laboratory of Viral Oncology, Aichi Cancer Center Research Institute, Chikusa-ku, Nagoya 464, Japan. Fax: (81) (52) 763 5233.

2. MATERIALS AND METHODS

2.1. *Chemicals*

5-Methylene-*bis*-2,6-dihydroxy-3-methyl-4-methoxyacetophenone (mallotophenone) and its three analogues (mallotochromene, mallotolerin and mallotojaponin) were isolated and purified from the pericarps of *Mallotus japonicus* Muell. Arg. (Euphorbiaceae) as described previously [10–12]. Structural formulae of these com-

pounds are shown in Fig. 1. MS-2 phage RNA was obtained from Boehringer, Mannheim, Germany. A primer DNA (18-mer; 5'-CTTCTTTGTTGTCTTCGA-3'), hybridizable with MS-2 RNA at 39 to 56 bases downstream of the coat protein gene, was chemically synthesized by a DNA synthesizer (ABI Co., USA). This oligonucleotide was mixed with MS-2 RNA at a molar ratio of 1:20 and annealed to make initiated MS-2 RNA as described previously [13]. The sources of other materials used in this work were as follows: [^{3}H]dTTP from Amersham International (Amersham, UK); unlabeled dTTP, poly(rA), oligo(dT) from P-L Biochemicals (Milwaukee, WI, USA); and DEAE-cellulose paper disc (DE81, diameter 23 mm) from Whatman (Springfield Mill, Maidstone, UK).

2.2. *HIV-reverse transcriptase*

HIV-1 reverse transcriptase was purified from *E. coli* harboring an expression plasmid for the precise coding sequence of the enzyme. Details of the purification procedures of p66 enzyme protein and some of its properties will appear elsewhere [14]. The purified enzyme was a generous gift from Dr. S.H. Wilson, NIH, USA.

2.3. *Assay for reverse transcriptase*

Reverse transcriptase activity was measured with each of $(rA)_n \cdot (dT)_{12-18}$ (reaction mixture A) and initiated MS-2 phage RNA (reaction mixture B) as the template·primer under the optimized reaction conditions for the respective template·primers. The reaction mixture A contained the following components: 50 mM Tris-HCl, pH 8.0; 3 μg/ml $(rA)_n \cdot (dT)_{12-18}$ (base ratio, 2:1); 10 μM [^{3}H]dTTP (400 cpm/pmol); 5 mM dithiothreitol; 50 mM KCl; 15% (v/v) glycerol; and 5 mM $MgCl_2$. The reaction mixture B included the following: 50 mM Tris-HCl, pH 7.5; 10 μg/ml initiated MS-2 RNA; 5 mM dithiothreitol; 2 mM $MgCl_2$; 1 μM each of dATP, dCTP, dGTP and [^{3}H]dTTP (6000 cpm/pmol); and 15% (v/v) glycerol. 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 [15].

3. RESULTS

3.1. *Effects of various phloroglucinol derivatives on the activity of HIV-reverse transcriptase*

Effects of mallotophenone and its analogues on the activity of HIV-reverse transcriptase were examined under the reaction conditions described in section 2. As shown in Fig. 2A, the enzyme activity was inhibited by 26, 38, 67 and 75% in the presence of 10 μg/ml mallotophenone, mallotolerin, mallotojaponin and mallotochromene, respectively, with $(rA)_n \cdot (dT)_{12-18}$ as the template·primer. The degree of inhibition was dose-dependent and more than 90% inhibition was achieved by mallotojaponin, for example, at a concentration of 25 μg/ml. The degrees of inhibition by mallotojaponin and mallotochromene are much stronger than those by mallotophenone and mallotolerin. The enzyme activity was also inhibited, though to lesser extent, by these compounds in the presence of initiated MS-2 phage RNA as the template·primer (Fig. 2B).

3.2. *Analysis of the mode of inhibition by mallotojaponin and determination of inhibition constant*

The mode of inhibition of reverse transcriptase activity by mallotojaponin was analyzed by changing the

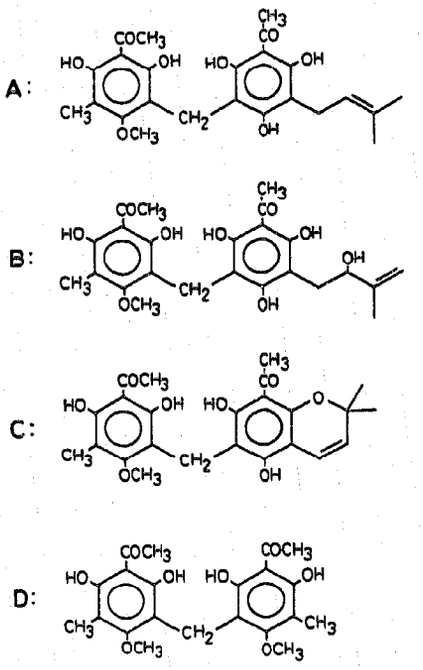

Fig. 1. Structural formulae of the four phloroglucinol derivatives examined in this study. A, mallotojaponin; B, mallotolerin; C, mallotochromene; and D, mallotophenone.

concentrations of either the template·primer, $(rA)_n \cdot (dT)_{12-18}$, or the triphosphate substrate, dTTP, in the presence of various concentrations of the inhibitor, mallotojaponin. As shown in Fig. 3, mallotojaponin inhibited the enzyme activity competitively with respect to the template·primer, $(rA)_n \cdot (dT)_{12-18}$, and non-competitively with respect to the triphosphate substrate, dTTP. The K_i value of the enzyme for

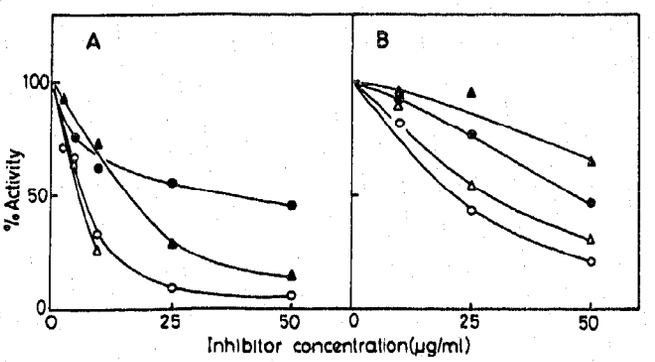

Fig. 2. Effects of four phloroglucinol derivatives on the activity of HIV-1 reverse transcriptase. Reverse transcriptase activity was measured with each of $(rA)_n \cdot (dT)_{12-18}$ (A) and initiated MS-2 phage RNA (B) under the conditions described in section 2 in the presence of various concentrations of each of the four phloroglucinol derivatives as indicated in the figure, by determining the incorporation of [^{3}H]dTMP. The compounds tested and the symbols used are: mallotojaponin (○), mallotolerin (●), mallotochromene (△), and mallotophenone (▲). The 100% values were 5.8 (A) and 2.7 (B) pmol.

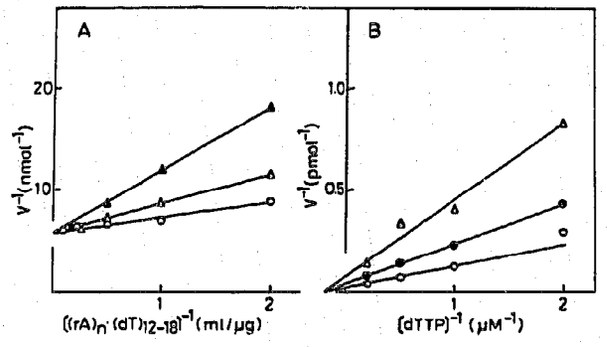

Fig. 3. Analysis of the mode of inhibition of HIV-1 reverse transcriptase activity by mallotojaponin. Reactions were carried out under the conditions described in section 2, except that various concentrations of $(rA)_n \cdot (dT)_{12-18}$ (A) and [^{3}H]dTTP (B) were used as the template · primer and the triphosphate substrate, respectively, in the presence of various concentrations of mallotojaponin. Mallotojaponin concentrations were 0 (○), 5 (●), 8 (△), and 12 (▲) μM.

mallotojaponin was determined to be 6.1 μM by replotting (Dixon plot) the data of Fig. 3A.

4. DISCUSSION

Four phloroglucinol derivatives examined in the present study have been isolated from pericarps of *Mallotus japonicus* which is a deciduous tree with red colored buds and is widely distributed in Japan [10–12]. Some parts of tree have already been used for a long time in folk medicine; the bark for treatment of ulcers and cancer, the leaves for boils. It was shown in previous reports [10–12] that these compounds are more or less cytotoxic to KB and L 5178Y cells in culture as well as to Ehrlich carcinoma, ascites and solid forms, in mice. To know the underlying mechanism(s) of the cytotoxicity, the effects of these compounds on various DNA polymerases have been tested and mallotojaponin was found to be a selective inhibitor of DNA polymerase β purified from KB cells (Nakane, et al., manuscript in preparation).

An extensive study has revealed that, of the four compounds tested, mallotojaponin and mallotochromene are particularly strong inhibitors for HIV-reverse transcriptase (Fig. 2). Differences in the inhibition potential among the four compounds seem to be due to the presence or absence and the nature of the side chains introduced (Fig. 1). The mode of inhibition of the enzyme activity by mallotojaponin was competitive with respect to the template · primer, $(rA)_n \cdot (dT)_{12-18}$, and non-competitive with respect to the triphosphate substrate, dTTP (Fig. 3). This type of inhibition is similar to those of suramin [16], baicalein [8] and other flavonoids [9], catechin derivatives [17] and asterriquinone and its analogues [18], indicating that all these cyclic compounds have the same inhibition mechanism for HIV-reverse transcriptase; i.e. the inhibitors interfere with the template · primer binding to the enzyme molecule. The inhibition was also observed when examined with initiated MS-2 phage RNA as a natural template · primer, indicating that the observed inhibition is not specific for the synthetic template · primer, $(rA)_n \cdot (dT)_{12-18}$.

Although mallotojaponin and mallotochromene are potent inhibitors of HIV-reverse transcriptase as described here, it is not yet clear whether these two compounds are effective as anti-HIV agents. It totally depends on whether and to what extent they inhibit HIV replication in intact cell culture system with susceptible human lymphocytes. Such tests are now in progress in our laboratories.

Acknowledgement: The authors are grateful to Mrs. S. Shinmura for preparing the manuscript.

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