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The inhibition of human immunodeficiency virus type 1 reverse transcriptase by avarol and avarone derivatives

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We have analyzed the effects of several natural compounds related to avarols and avarones on the catalytic functions of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT). The most potent substances, designated as avarone A,B and E and avarol F, inhibited indiscriminately the enzymatic activities of HIV-1 RT, namely the RNA-dependent and DNA-dependent DNA polymerase as well as the ribonuclease H. The inhibition of the DNA polymerase activity was found to be non-competitive with respect to either the template-primer or the deoxynucleotide-triphosphate. These studies suggest that the hydroxyl group at the ortho position to the carbonyl group at the quinone ring is involved in blocking the RT activity. The identification of the active site of the inhibitors will hopefully lead to the rational design of new potent anti-HIV drugs.

AIDS; HIV-1; Reverse transcriptase; Inhibitor; Avarol; Avarone

1. INTRODUCTION

Active inhibitors of human immunodeficiency virus (HIV) may be suitable for the chemotherapy of acquired immunodeficiency syndrome (AIDS), the devastating human disease caused by HIV [1,2]. One of the ideal specific targets for chemotherapeutic treatment of HIV is the viral encoded enzyme reverse transcriptase (RT). This enzyme has a key role in the early stages of HIV infection and is responsible for converting the viral genomic RNA into proviral double-stranded DNA which is subsequently integrated into the host chromosomal DNA. Reverse transcriptases are unique to retroviruses and no cellular homologues are known (except those associated with endogenous retroviruses and retrotransposons). The search for anti-HIV RT drugs and a detailed study on the mechanism of the molecular and catalytic properties of reverse transcriptase (thus contributing towards the design of new drugs) require the availability of relatively large quantities of purified active protein. For this reason, several recombinant RT expression systems have been developed in bacteria, leading to the synthesis of substantial amounts of highly active, soluble and nearly authentic HIV-1 RT [3–5]. This recombinant enzyme is in many catalytic features indistinguishable from the enzyme found in virions [3,6].

Over the past few years, large scale screening of marine species have produced a number of exciting leads towards finding new pharmaceutically useful agents. In direct response to these encouraging results, we have decided to screen different marine natural products for their anti-HIV-RT activities. The presence of large amounts of recombinant HIV-1 RT combined with the convenient accessibility of various natural products, isolated from the Red Sea fauna (mainly sponges and corals) have enabled us to conduct a wide survey for specific inhibitors of this enzyme. During the course of this study we found that three novel natural products derivatives of the sesquiterpenoid avarol, isolated from the marine sponge *Dysidea cinerea* Keller from the Gulf of Eilat in the Red Sea, are potent in vitro inhibitors of HIV-1 RT.

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2. MATERIALS AND METHODS

Avarol and avarone derivatives designated as A–F as shown in the structural formulas in Fig. 1 were a generous gift of Professor Y. Kashman. Apart from avarone A that is a known compound, the other 4 are novel secondary proferan metabolites (Professor Kashman, personal communication). The substances were dissolved in 100% dimethylsulphoxide (DMSO) to final concentrations of 10 mg/ml. The final DMSO concentration in the enzymatic assays was 1%, a concentration that did not affect the different RT-associated activities.

HIV-1 reverse transcriptase was a recombinant protein expressed in *E. coli* with an apparent molecular weight of 66 kDa (that differs from the RT found in HIV-1 only in two additional amino terminal amino acids residues [3]). The HIV-1 RT was purified to homogeneity according to Clark et al. [7].

2.1. *Enzyme assays*

In all enzymatic reactions mixtures the enzymes were preincubated with or without the various inhibitor concentrations for 5 min at 30°C. The enzymatic reactions were initiated by adding the ap-

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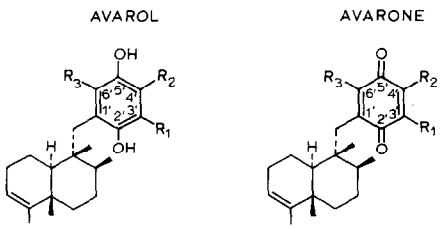

		R_1	R_2	R_3
A	3'-hydroxy avarone	OH	H	H
B	4'6'-dihydroxy avarone	H	OH	OH
C	6'-acetoxy avarol	H	H	OAc
D	6'-acetoxy avarone	H	H	OAc
E	6'-hydroxy-4'methoxy avarone	H	OMe	OH
F	6'-hydroxy avarol	H	H	OH

Fig. 1. Structural formulas of avarol and avarone derivatives.

propriate substrate and subsequently incubated at 37°C for 30 min. Inhibition of enzyme activity was calculated relative to the linear reaction rates observed where no drug was added. The definition of the enzymatic activities is as follows: One unit of DNA polymerase activity is defined as the amount of enzyme that catalyzes the incorporation of 1 pmol of dGTP into the DNA product after 30 min at 37°C, under the standard assay conditions (see below). One unit of the RNase H activity is the amount of enzyme that catalyzes the hydrolysis of 1 pmol AMP after 30 min at 37°C, under the assay conditions.

RNase H assay was performed by measuring the release of radioactively labelled molecules from [^{3}H]poly(rA)·poly(dT) into the trichloroacetic acid soluble fraction. The assay was carried out in a final volume of 0.1 ml containing 50 mM Tris-Cl (pH 8.0), 50 mM KCl, 8 mM $MgCl_2$, 2.5 mM dithiothreitol and approximately 110 pmol of total nucleotides of [^{3}H]poly(rA)·poly(dT). The reactions were terminated by transfer onto ice followed by the addition of 250 μg herring sperm carrier DNA and 0.6 ml 10% (w/v) ice-cold TCA. After 5 min on ice the solutions were centrifuged at 10000 × *g* for 5 min and the supernatants were counted. The substrate [^{3}H]poly(rA)·poly(dT) was prepared according to the procedure described by Hizi and Joklik [8].

RT-associated DNA polymerase assays were carried out in a final volume of 0.1 ml containing 25 mM Tris-Cl (pH 8.0), 40 mM KCl, 8 mM $MgCl_2$, 3 mM dithiothreitol. For determining the RDDP activity, poly(rC)·oligo(dG), at a final concentration of 0.1 units/ml, was used as a primer-template and labelled deoxynucleotide triphosphate was [^{3}H]dGTP (spec. act. 1100–1300 cpm/pmol) at a final concentration of 5 μM. For HIV-1 RT DDDP assay activated herring sperm DNA (prepared according to Spanos et al. [9]) was used as the transcribed DNA at a final concentration of 20 μg/ml. The three unlabelled deoxynucleotides triphosphates dTTP, dATP and dCTP, were used at final concentrations of 50 μM each and [^{3}H]dGTP was at 5 μM (spec. act. 1110–1300 cpm/pmol).

3. RESULTS

Substances that inhibit in vitro the unique HIV-RT catalytic activities are likely to fall into one of three different categories: (i) Compounds that block all RT-catalytic activities, i.e., the RNA-dependent DNA polymerase (RDDP) and DNA-dependent DNA polymerase (DDDP), as well as the ribonuclease H (RNase H) activities associated with retroviral reverse transcriptases. (ii) Inhibitors of the DNA polymerases exhibiting little or no effect on the RNase H function. (iii) Compounds that block the RNase H activity without significantly affecting the DNA polymerase function. In the present study we have chosen to examine the effects of several marine natural products of the avarol and avarone group (see structural formulas in Fig. 1) on the different catalytic functions of HIV-1 RT. Compounds defined as effective inhibitors were those found to inhibit more than 50% of either one of the catalytic activities associated with HIV-1 RT at a final inhibitor concentration of either 10 μg/ml (for RDDP and DDDP activities) or 50 μg/ml (for RNase H). As can be seen in Table I, 4 of these compounds, avarone A (3'-hydroxy avarone), avarone B (4',6'-dihydroxy avarone) and avarone E (6'-hydroxy-4'-methoxy-avarone) and avarol F (6'-hydroxy avarol) were found to inhibit HIV-1 RT associated RDDP. RDDP activity was impaired by more than 70% of its initial activity in the presence of the derivatives designated A, B and F, and by 91% in the presence of avarone E. The IC_{50} values (inhibitor concentrations yielding 50% inhibition of the enzymatic activities) determined from the dose-dependent curves were approximately 6.8, 5.0, 1.0 and 7.0 μg/ml for avarones A, B, E and avarol F, respectively. Contrariwise, the two derivatives, avarol C (6'-acetoxyavarol) and avarone D (6'-acetoxyavarone), were devoid of any significant activity against HIV-1 RT associated RDDP (Table I). Avarones A and B exhibited only a moderate activity against RT-associated RNase H (54% and 41% inhibition at 50 μg/ml, respectively) whereas avarone E and avarol F effectively inhibited this activity (by 94% and 100%, respectively, at 50 μg/ml inhibitor). Considered

Table I

The effect of different avarol and avarone derivatives on HIV-1 RT-associated RDDP and RNase H activities

	HIV-1 RT enzymatic activity (%)		
	RDDP[a]		RNase H
Avarone A	27	(6.8)	46
Avarone B	30	(5)	59
Avarol C	100	(≫50)	86
Avarone D	98	(≫50)	96
Avarone E	9	(1.0)	6
Avarol F	28	(7.0)	0

[a] The figures in parentheses are the IC_{50} values calculated for the inhibition of HIV-1 RT-associated RDDP activity by the different compounds. These values express the inhibitor concentrations in μg/ml

The inhibition of RDDP and RNase H activities by the different avarones and avarols were performed at concentrations of 10 μg/ml and 50 μg/ml, respectively. All residual activities are expressed as a percentage of the activities of the control samples with no inhibitor. Activity of 100% corresponds to 125 units and 50 units of enzyme for RDDP and RNase H, respectively

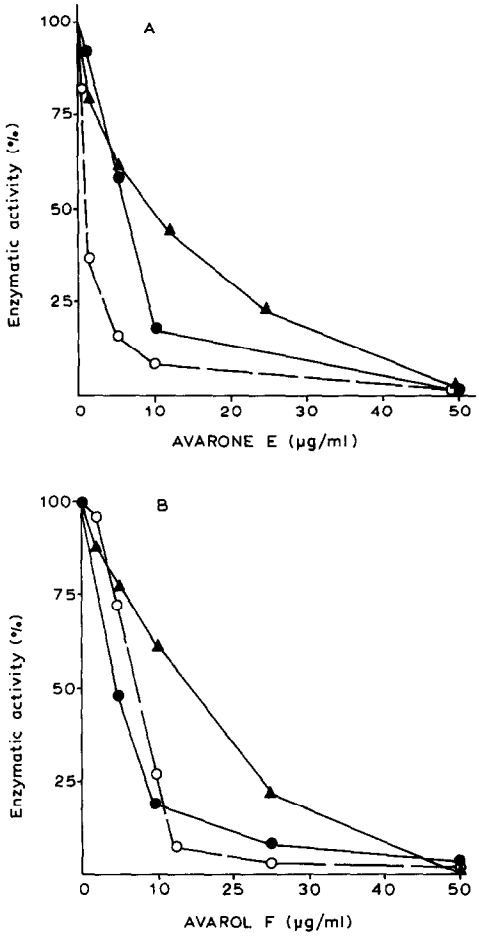

Fig. 2. Dose–response curves of HIV-1 RT inhibition by avarone E (A) and avarol F (B). Assay conditions are as described in section 2. The 100% enzymatic activity for RDDP (○), DDDP (●) and RNase H (▲) activities correspond to 120, 10 and 55 units, respectively.

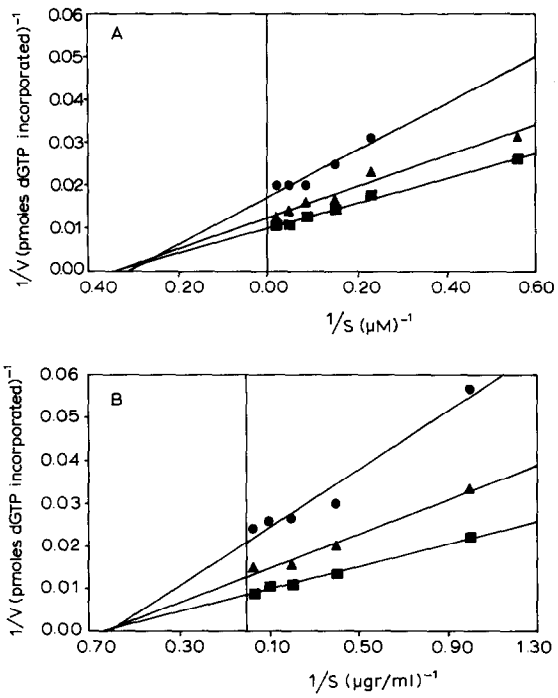

Fig. 3. Double reciprocal plots of the effect of increasing concentrations of both dGTP (A) and the primer-template poly(rC)·oligo(dG) (B) on the incorporation of [^{3}H]dGTP by HIV-1 RT, in the absence (■) or in the presence of 2 μg/ml (▲) and 4 μg/ml (●) of avarone E. The reactions were carried out for 10 min at 37°C under the conditions described in section 2.

together, it is apparent that avarone E and avarol F are the most potent inhibitors out of the 6 derivatives analyzed. Therefore, these two compounds were selected for further study as representatives of the active inhibitors. We have measured the extent of the inhibition of the three catalytic activities associated with HIV-1 RT as a function of inhibitor concentrations. As can be seen in Fig. 2A, B, all three functions associated with HIV-1 RT (i.e., RDDP, DDDP and RNase H) were susceptible to the inhibitory effects of avarone E, and avarol F. The IC_{50} values determined from these dose response curves for each enzymatic activity were in the case of avarone E 1 μg/ml, 6 μg/ml and 14 μg/ml and in the case of avarol F 7 μg/ml, 4.5 μg/ml and 14.5 μg/ml for RDDP, DDDP and RNase H activities, respectively.

3.1. *Analysis of the mode of inhibition and determinations of kinetic constants*

We have studied the kinetics of inhibition of the most potent inhibitor avarone E by analyzing the initial rates of the DNA polymerizing reactions as a function of increasing concentrations of either the template-primer or the deoxynucleoside-triphosphate substrate (in the absence or in the presence of inhibitor at final concentrations of 4 or 8 μg/ml. The values of V_{max} and K_m were determined from the double-reciprocal plots of velocity rates versus substrate concentrations (Fig. 3). The K_m values for avarone E were calculated to be 2.8 μM for dGTP and 1.6 μg/ml for poly(rC)·oligo(dG) irrespective of the presence of the inhibitor, whereas the V_{max} values were suppressed as a function of the presence of avarone E. Therefore, the mode of inhibition of RT activity by avarone E is noncompetitive with respect to both dGTP and primer-template, i.e., reduced V_{max} and unaltered K_m values. Thus, the results suggest that avarone E binds HIV-1 RT molecules at sites different from the binding sites of either one of the substrates for DNA synthesis.

4. DISCUSSION

In the scientific effort to develop anti-AIDS drugs, a considerable number of compounds with diverse molecular properties have been so far tested for their

anti-HIV RT activities. These include deoxynucleoside analogues (such as 3′-azidothymidine-AZT), or 2′,3′-dideoxynucleosides (such as dideoxynosine or dideoxycytidine) that inhibit the viral RT in the form of 5′-triphosphonucleosides [10–14], foscarnet [15], suramine [16], rifabutine [18], or HPA 23 [18]. Unfortunately, some of the drugs were found to be either insufficiently potent or too toxic to be considered for further development as anti-HIV drugs. To date, the only drug that was approved for clinical use is AZT, despite its known side effects [19]. The emergence of AZT-resistant HIV strains in patients treated with the drug has serious implications when considering it for wider use in the future [20]. This highlights the urgent need for the development of alternative drugs against AIDS.

In the course of screening for novel natural products with anti-HIV-1 RT activities we have found that compounds related to avarol and avarone were effective inhibitors. Avarol and avarone were previously reported to possess a variety of biological activities such as: (i) a potent antileukemic activity both in vivo and in vitro [21]; (ii) T-lymphotropic cytostatic activity in vitro [22]; (iii) anti-HIV activity in vitro in the H-9 cell system [23]. The results presented in this communication reveal another facet of the biological activities associated with this group of natural substances. Four avarol and avarone derivatives designated A, B, E, F, out of which B, E and F are novel secondary metabolites (Kashman et al., personal communication), were found to be potent inhibitors of HIV-1 RT in vitro. Avarone A and avarol F inhibited the enzyme activities most effectively. The two derivatives with 6′-acetyl-substituents (avarol C and avarone D) were devoid of any significant inhibitory activity against reverse transcriptase. This is in line with the results previously reported for unmodified avarol and avarone (also lacking the 6′-hydroxyl group) that exhibited no significant inhibitory activities against MuLV and HIV-1 RT DNA polymerase functions as well as against mammalian DNA polymerase [24]. When taken together, it seems that the hydroxyl group at the *ortho* position to the carbonyl group of the quinone ring (as in the case of the derivatives A, B, E and F) is a requisite for inhibitory activity of these compounds. Similarly, we have recently found that in the case of another natural sesquiterpenoid, illimaquinone, the inhibitory site of the compound is an hydroxyl group at the *ortho* position to the carbonyl group of the quinone ring. However, in this case the inhibition effect was restricted mainly to the RNase H function of HIV-1 RT (submitted). Considered together, we believe that the identification of the inhibitory site of a compound is an important step towards the rational design of new potent anti-HIV and possibly anti-AIDS drugs.

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