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Inhibition mechanisms of HIV-1, Mo-MuLV and AMV reverse transcriptases by Kelletinin A from *Buccinulum corneum*

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Abstract. Kelletinin A [ribityl pentakis (p-hydroxybenzoate)] (KA), an inhibitor of HTLV-1 replication isolated from *Buccinulum corneum*, showed a noncompetitive inhibitory activity with respect to the template primer and to dTTP in the poly(rA)·oligo(dT)$_{12-18}$-directed reaction of HIV-1, Mo-MuLV and AMV reverse transcriptases (RT). Analysis of natural and synthetic KA-related compounds showed that the inhibitory activity was strictly related to the structural peculiarities of the molecule. In the presence of DNA as template primer the inhibition mechanism was drastically modified: HIV-1 RT activity was stimulated by low concentrations of KA and was inhibited by increasing the concentration of the compound, while Mo-MuLV and AMV activities were irreversibly inhibited by the formation of a non-reactive complex. The RNase H activities of these RTs were not affected by KA. The results of this study suggest a different mechanism of interaction of Kelletinins with HIV-1 RT compared with other non-nucleoside inhibitors. A possible use of these drugs in combination therapy and in the design of structure-based reverse transcriptase inhibitors is discussed.

Key words. Kelletinin A; human immunodeficiency virus type 1 (HIV-1); Moloney murine leukemia virus (Mo-MuLV); avian myeloblastosis virus (AMV); reverse-transcriptases; inhibition.

The reverse transcription of viral genomic RNA to double stranded DNA by reverse transcriptase (RT) plays a crucial role in the progression of retroviral infections[1,2]. RT exhibits three enzymatic activities: the RNA-dependent DNA-polymerase (RDDP), responsible for reverse transcription of genomic RNA (+) strand into DNA (−) strand, the RNase H activity that hydrolyzes the RNA moiety of the RNA-DNA heteroduplex, and the DNA-dependent DNA-polymerase (DDDP) that converts the DNA (−) strand to double stranded DNA.

The native RT of the human immunodeficiency virus type 1 (HIV-1), the etiological agent of acquired immunodeficiency syndrome (AIDS), is a heterodimer composed of two peptides of 66 and 51 kDa. The 66 kDa subunit exhibits the two DNA polymerase activities in the N-terminal domain and the RNase H activity in the C-terminal domain. The p51 is derived from proteolytic cleavage of the C-terminal domain of the 66 kDa peptide by HIV-1 protease and plays an allosteric role by stabilizing and activating the 66 kDa subunit[2–6]. At the present time, the enzyme is available from a bacterial recombinant systems and information about its structural and catalytic properties is increasing[7–17]. HIV-1 RT inhibitors are classifiable in two main categories (for rewiew see ref. 10): nucleoside analogs (such as AZT, ddI etc.) that act as chain terminators or competitive inhibitors for the dNTP-binding site, and non-nucleoside inhibitors (also referred to as TIBO-like inhibitors[18]) whose mechanism of action is not well understood. Clinical trials of some of these RT inhibitors have revealed accompanying toxicity[19,20] or the emergence of drug-resistant viral strains[10,21] as a result of genomic hypermutability during HIV-1 replication.

The RTs from avian myeloblastosis virus (AMV) and Moloney murine leukemia virus (Mo-MuLV) are composed of two structurally related subunits of 63 kDA (α) and 95 kDA (β) or by a single subunit of about 80 kDA, and like HIV-1 RT exhibit the three enzymatic activities organized into two distinct domains[1,2].

Recently we reported that Kelletinin A [ribityl pentakis (p-hydroxybenzoate)] (KA), a natural compound isolated from the marine gastropod *Buccinulum corneum*[22], showed antiviral activity in human T-cell leukemia virus type-1 (HTLV-1)-infected MT2 cells and interfered with viral transcription, as shown by reduction of the levels of high molecular weight transcripts[23]. Furthermore, the compound inhibited partially purified HTLV-1 reverse transcriptase *in vitro*.

The present paper describes the mechanism of inhibition of HIV-1, Mo-MuLV and AMV RTs by Kelletinin A and some related compounds.

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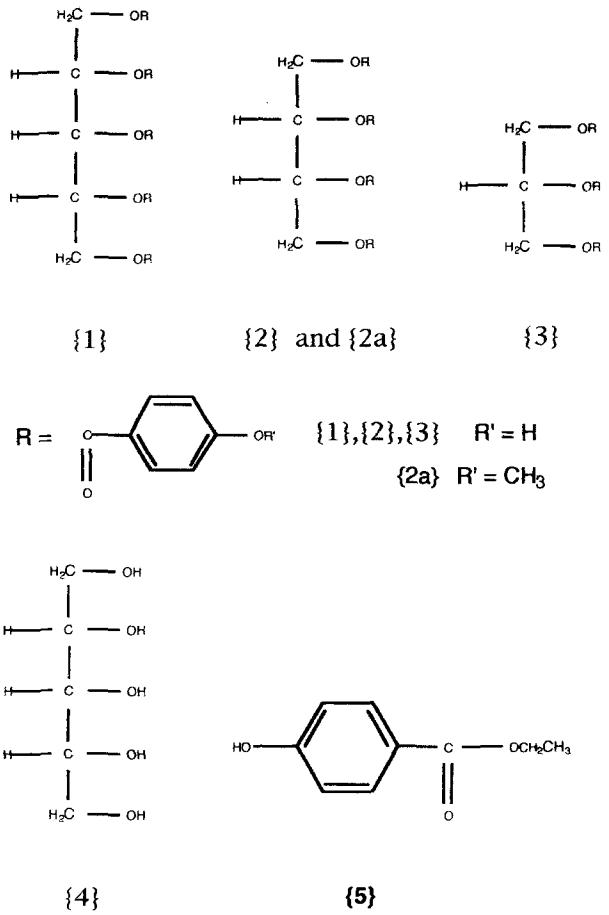

Figure 1. Structural formulae of KA and related compounds.

Materials and methods

Kelletinin A and related compounds. KA {**1**} [ribityl pentakis (p-hydroxybenzoate)] and KI {**2**} [erythrityl tetrakis (p-hydroxybenzoate)] were purified according to Cimino et al.[22]; glyceryl trikis (p-hydroxybenzoate) {**3**} and methoxy-KI {**2a**} were synthesized, purified and characterized as previously described[24] (structural formulae are shown in fig. 1). The compounds were dissolved in 100% dimethylsulphoxide at a maximum concentration of 25 mM. The final concentration of dimethylsulfoxide in the assays did not exceed 0.2% (v/v); this concentration did not affect the different RT-associated enzymatic activities.

Enzymes and other chemicals. HIV-1 RT (3,000 U/mg) was obtained from American Bio-Technologies Inc. (Cambridge, MA, USA); Moloney murine leukemia virus (Mo-MuLV) RT (57,576 U/mg) and avian myeloblastosis virus (AMV) RT (54,500 U/mg) were purchased from Pharmacia Biotech Inc. (Milwaukee, WI, USA); Superscript™ (200 U/μl), a modified RT produced from a cloned Mo-MuLV gene from which the RNAse H sequence has been deleted[25], was from Gibco-BRL; [^{3}H] dTTP (sp. act. 30 Ci/mmol), [^{3}H] dATP (sp. act. 19 Ci/mmol) and [^{3}H] poly(rA) (sp. act. 532 mCi/mmol. of nucleoside residues) were from Amersham Int. (Buckinghamshire, UK); poly(rA)·oligo(dT)$_{12-18}$ and poly(dT) were purchased from Pharmacia Biotech Inc. (Milwaukee, WI, USA); salmon sperm DNA (Sigma Aldrich (Milan, Italy)) was activated as previously described[26]; DNase/RNase-free bovine serum albumin (BSA) was from Pharmacia; and tRNA mixture was from Boehringer (Mannheim, Germany).

Enzyme assay. RNA-dependent DNA-polymerase activity was assayed at 37 °C, in a reaction mixture (100 μl) containing 50 mM Tris-HCl buffer (pH 7.8), 40 mM KCl, 8 mM $MgCl_2$, 3 mM dithiothreitol (DTT), 100 μg/ml of BSA, 30 μg/ml of poly(rA)·oligo(dT)$_{12-18}$, 50 μM [^{3}H] dTTP (0.05–0.25 μCi/nmol) and 20 to 200 ng of HIV-1 RT or 3 to 30 ng of Mo-MuLV RT and of AMV RT. The reaction was started by addition of [^{3}H] dTTP and $MgCl_2$ to the pre-warmed mixture and was stopped by addition of 1 ml of cold 10% trichloroacetic acid (TCA) together with 2 ml of cold 0.1 M sodium pyrophosphate and 100 μg of tRNAs. The acid precipitable product was collected on GF/C filters (Whatman Biochemicals Ltd. (Springfield Mill, Kent, UK)); the filters were washed with 5% cold TCA followed by 70% ethanol, then dried and radioactivity measured by scintillation counting.

DNA-dependent DNA-polymerase activity was assayed at 37 °C in a reaction mixture (100 μl) containing 50 mM Tris-HCl buffer (pH 8), 8 mM $MgCl_2$, 3 mM DTT, 40 mM KCl, 100 μM each of dGTP, dCTP and dTTP (Sigma), 50 μM [^{3}H] dATP (0.25–1 μCi/nmol), 0.5 mg/ml of activated DNA, 100 μg/ml of BSA and 50 to 200 ng of HIV-1 RT. The reaction was processed as before.

RNase H activity was assayed at 37 °C in a reaction mixture (100 μl) containing 40 mM Tris-HCl buffer (pH 8), 15 mM $MgCl_2$, 0.02% Nonidet P-40, 100 μg/ml BSA, 0.2 μCi of [^{3}H] poly(rA), 0.4 μg of poly(dT) and 0.2–0.5 μg of HIV-1 RT. [^{3}H] poly(rA) and poly(dT) were previously annealed in 10 mM Tris-HCl buffer (pH 8) containing 1 mM EDTA by heating at 55 °C for 5 min and then cooling to room temperature. The reaction, linear with respect to enzyme concentration (0.25–1 μg) and time up to 30 min, was stopped by adding 100 μl of cold 20% TCA and 200 μg of carrier DNA. Samples were chilled in ice for 10 min and centrifuged at 10,000 × g for 10 min in swinging rotor buckets (HB4 Sorvall) and the supernatant was subjected to scintillation counting.

Results

Effect of Kelletinin A and related compounds on poly(rA)·oligo(dT)$_{12-18}$-directed reaction of HIV-1 Mo-MuLV and AMV RT. The effect of KA on the time course of poly(rA)·oligo(dT)$_{12-18}$-directed reaction of HIV-1 RT is shown in figure 2A. The rate of polymerase reaction

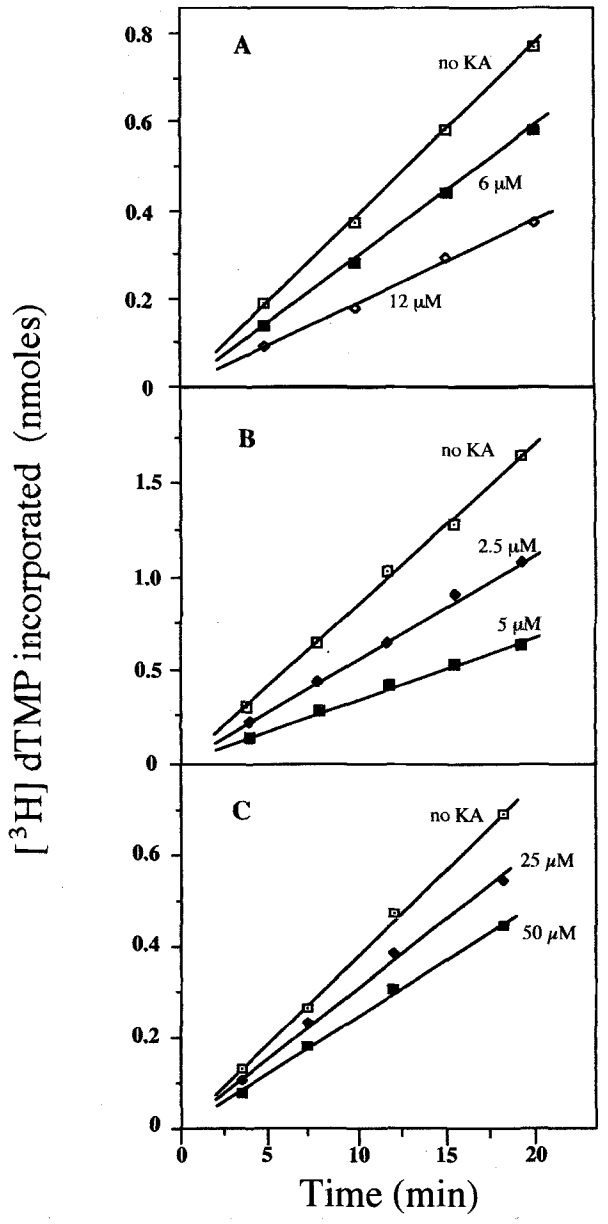

Figure 2. Time course of poly(rA)·oligo(dT)$_{12-18}$-directed reactions of HIV-1 RT (panel A), Mo-MuLV RT (panel B) and AMV RT (panel C). Each assay contained 130, 14, or 7 ng of HIV-1, Mo-MuLV, and AMV RTs, respectively.

in the presence of the inhibitor was linear with time and decreased in proportion to the amount of compound added. This mode of inhibition was also observed for Mo-MuLV (fig. 2B) and AMV RT (fig. 2C).

Natural and synthetic KA-related compounds were tested on poly(rA)·oligo(dT)$_{12-18}$-directed reaction of HIV-1 RT, and the results are reported in table 1. Among these compounds (formulae are shown in fig. 1), only the natural products KA {**1**} and KI {**2**} inhibited the enzyme and did so to the same extent. The inhibitory properties of KA and KI are believed to be strictly related to the structural properties of the molecules, due to the following considerations: 1) neither p-hydroxy-ethylbenzoate {**5**} nor rybitol {**4**} are inhibitors of HIV-1 RT; 2) the hydroxyl groups of the p-hydroxybenzoate residues are involved in the inhibition process, because the etherification of these groups caused a complete loss of inhibitory activity as in {**2a**}; 3) molecular size appeared to be critical for the inhibitory effect of the Kelletinins. In fact, glyceryl trikis (p-hydroxybenzoate) {**3**}, the lower homologue of KI, tested up to the maximal concentration obtainable in aqueous media, did not inhibit significantly the RT activity.

Table 1. Effect of Kelletinin A and related compounds on poly(rA)·oligo(dT)$_{12-18}$-directed reaction of HIV-1 RT

		Residual activity %
Kelletiin A {**1**}	6 μM	75%
	12 μM	54%
	25 μM	26%
	50 μM	<1%
Kelletinin I {**2**}	12 μM	56%
	25 μM	30%
	50 μM	<1%
Glyceryl trikis {**3**}	25 μM	92%
	50 μM	87%
Rybitol {**4**}	up to 200 μM	100%
p-Hydroxy-ethylbenzoate {**5**}	up to 50 μM	100%
Kelletinin I methoxy-derivative {**2a**}	up to 25 μM	100%

The assays were performed in triplicate using 130 ng of HIV-1 RT under conditions of linearity. All residual activities are expressed as percentage of the activities of the control samples. Activity of 100% corresponded to 0.78 nmol of dTTP incorporated; the standard deviation did not exceed 8%.

Due to its greater solubility in aqueous media, more extensive studies were performed on KA.

Analysis of the mode of inhibition. In order to establish the kinetic mechanism of KA inhibition, we analyzed the initial rates of the polymerase reaction with and without inhibitor, as a function of increasing concentration of one of the two substrates [poly(rA)·oligo(dT)$_{12-18}$ or dTTP] in the presence of saturating concentration of the other. Figure 3A shows a Lineweaver-Burk plot analysis of the changes in the initial rate of the reaction as a function of increasing concentration of the template-primer. KA acted as a noncompetitive inhibitor: i.e., in its presence the apparent K_m (1.8 μg/ml) was not affected while the V_{max} was lowered. A noncompetitive mode (fig. 3B) was also observed in the analysis of initial velocity changes as a function of increasing dTTP concentration (apparent K_m 10.7 μM). A K_i of 13 μM was calculated by Dixon's plot analysis. The same analysis, performed using Mo-MuLV and AMV RT, provided values of K_i of 3.2 μM and 60 μM, respectively. These inhibition constants, as expected for a pure noncompetitive inhibitor, were comparable to IC_{50} values calculated from the data shown in figure 2 and other data not shown.

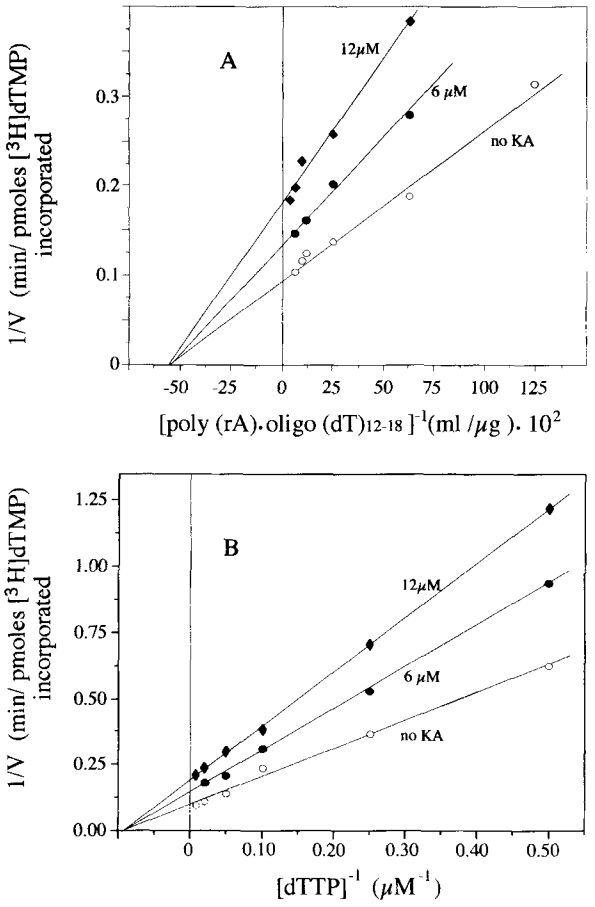

Figure 3. Lineweaver-Burk plot analysis of the effect of increasing poly(rA)·oligo(dT)$_{12-18}$ (panel A) and dTTP (panel B) concentrations on the initial rates of RNA-dependent HIV-1 RT reaction. The assays were performed using 30 ng of the enzyme at the indicated KA concentrations.

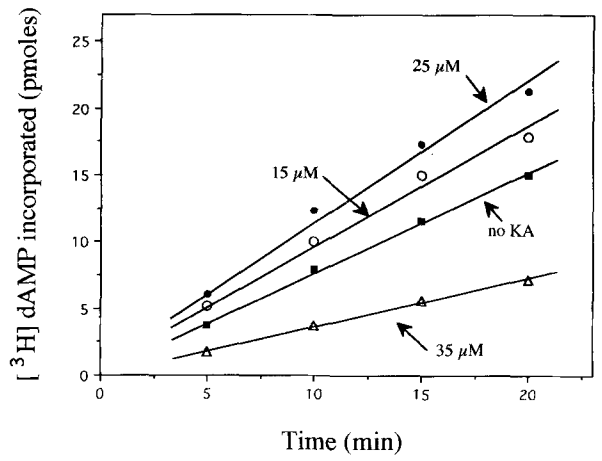

Figure 4. Time course of DNA-directed reaction of HIV-1 RT. The assays were performed as described in 'Materials and methods', in the presence of the indicated KA concentration, using 50 ng of enzyme.

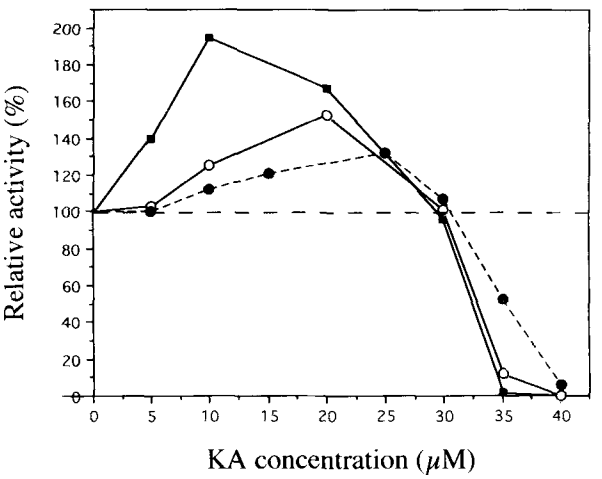

Figure 5. Effect of KA on DNA-directed reaction of HIV-1 RT. The enzyme (50 ng) was assayed in the presence of 50 (—■—), 150 (—○—) or 500 (—●—) μg/ml of activated DNA. Relative activities were calculated by comparing, for each DNA concentration, the velocity of the reaction in the presence of KA to that of a control reaction performed in absence of the drug; the coefficients of linear correlation were greater than 99%. The experiments were performed in triplicate and the standard deviation did not exceed 5% of the mean value.

Effect of KA on DNA-directed and RNase H activities of HIV-1, Mo-MuLV and AMV RT. KA exerted a dual modulatory action on the DNA-directed DNA-polymerase activity of HIV-1 RT. In fact, in the presence of saturating concentrations of DNA and deoxynucleotide triphosphate, the reaction was activated by low concentrations of KA and was inhibited by increasing the concentration of the compound (fig. 4). Furthermore, at the same KA concentration, the degree of activation or inhibition was dependent on DNA concentration (fig. 5) and independent of deoxynucleotide triphosphate concentration (data not shown). Under conditions of activated reaction, analysis of the initial velocity as a function of increasing concentration of DNA showed that the most relevant effect of the compound was the decrease in the apparent K_m of HIV RT for the DNA (fig. 6).

The time course of DDDP reaction of Mo-MuLV and AMV RT is shown in figure 7A and B respectively. The rate of the reaction for both enzymes in the presence of KA was linear during the first minutes and then declined with time, reaching a plateau. The enzymatic activity was not restored by addition of DNA or deoxynucleotide triphosphates or magnesium ions to the reaction mixture (data not shown). Moreover, the DDDP activity of AMV RT was inhibited by KA to a greater extent than the RDDP activity, with IC_{50} values of 10 and and 60 μM respectively (these values were calculated in the range of linearity of the two reactions).

Finally, KA did not significantly affect the RNase H activity of HIV-1 and AMV RTs up to a concentration of 50 μM (table 2).

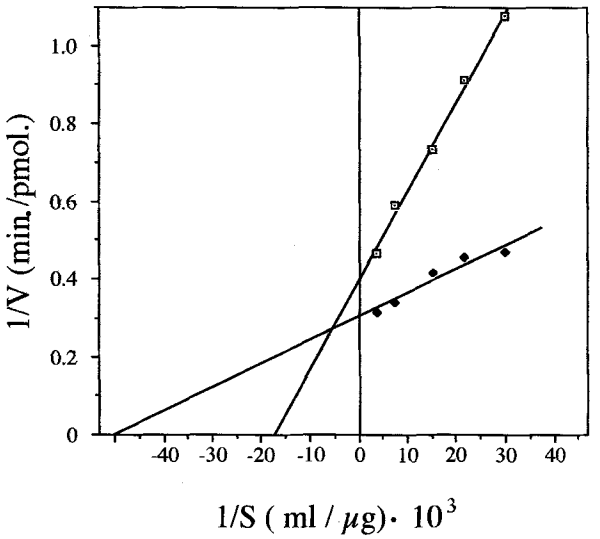

Figure 6. Lineweaver-Burk plot showing the effect of DNA concentration on the initial velocity of DNA-directed HIV-1 RT reaction. The assays were performed using 50 ng of enzyme in the presence (—◆—) or absence (—□—) of 10 μM KA.

Discussion

The data reported in this paper demonstrate that KA interacts in different ways with the two DNA polymerizing activities of HIV-1, Mo-MuLV and AMV RT and that the mechanism of inhibition is strictly related to the template primer utilized (i.e. poly(rA)·oligo(dT)$_{12-18}$ or 'activated' DNA).

KA acted as a noncompetitive inhibitor of RDDP activity of the three enzymes with respect to poly(rA)·oligo-(dT)$_{12-18}$ and dTTP. According to a reaction scheme recently proposed[15], these kinetic data could be explained assuming that KA does not interfere with the sequential and ordered binding of the two substrates. In other words, the compound is able to bind reversibly all the enzyme forms present at equilibrium, thus affecting only the catalytic step of the reaction or its processivity. Furthermore, this inhibition was strictly dependent both on the three-dimensional structure of Kelletinins and on the presence of the aromatic hydroxyl groups.

In the presence of DNA, the mechanism of inhibition is dramatically modified. In fact, DNA-dependent activity of HIV-1 RT was stimulated by low KA concentrations and this effect was correlated to an apparent increase of enzyme affinity for the template primer. By increasing KA concentration, the reaction was reversibly inhibited and the degree of inhibition was dependent on DNA concentration. Differences in the inhibition mechanism with respect to the secondary structure of the template primer have been described for the class of non-nucleoside inhibitors of the HIV-1 RT[27-33].

Furthermore, KA did not affect the RNase H activity of HIV-1 and AMV RT but inhibited the two DNA polymerase activities of a truncated form of Mo-MuLV RT lacking the RNAse H domain[25]. The data support the hypothesis that the compound, as described for other non-nucleoside inhibitors, could bind to the N-terminal polymerase domain of HIV-1 RT[10]. This domain is

Table 2. Effect of KA on RNase-H activities of HIV-1 and AMV RTs.

	^{3}H dAMP released (pmol/min)	
	HIV-1 RT	AMV RT
No addition	0.72 (100%)	1.32 (100%)
KA 10 μM	0.64 (88%)	-
25 μM	0.58 (80%)	1.33 (101%)
50 μM	0.59 (82%)	1.35 (102%)

The assays were performed as described in methods using 0.5 μg of HIV-1 or AMV RTs.

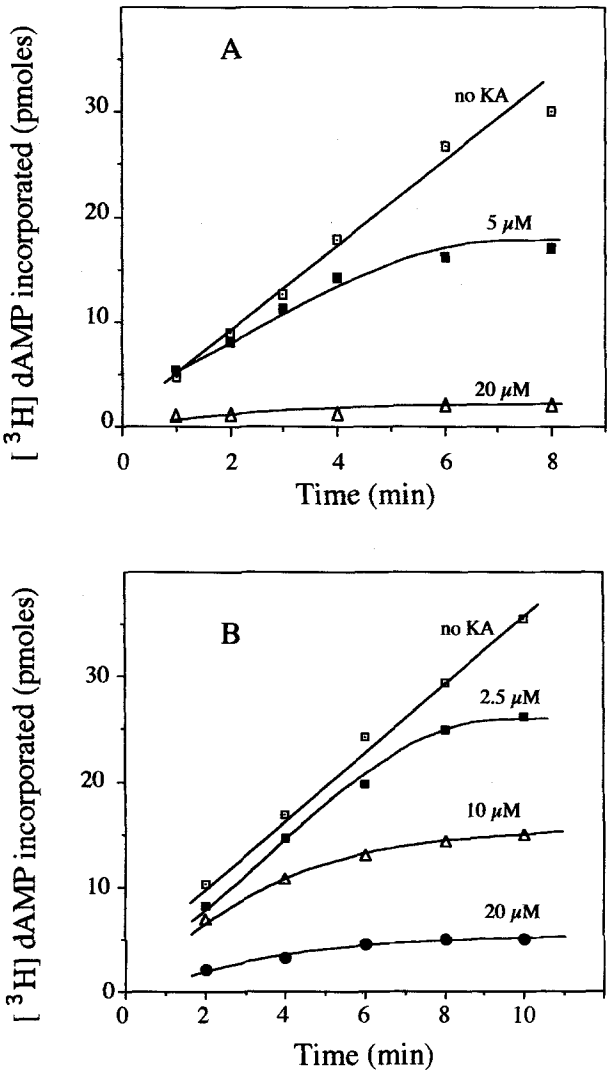

Figure 7. Time course of DNA-directed reaction of Mo-MuLV Superscript™ RT (panel A) and AMV RT (panel B). The assays were performed as described in 'Materials and methods', in the presence of the indicated KA concentration, using 5 RT units of Superscript™ or 50 ng of AMV RT.

constituted by four subdomains, called 'fingers', 'palm', 'thumb' and 'connection', organized to form a cleft (reminiscent of the cleft in the pol domain of Klenow fragment of *E. coli* DNA pol I) in which the template primer binds[34]. The available data do not allow an exhaustive description of the interaction(s) of KA with HIV-1 RT and more detailed experiments, including X-ray crystallographic analysis, would be necessary to clarify the mechanisms of action. We can hypothesize that the binding of KA to a site (not involved per se in the binding of dNTP and template primer as demonstrated by the reported data) of one of the subdomains of HIV-1 RT polymerase domain could induce conformational modifications that affect the alignment of the template primer in the cleft. These modifications could influence in a negative or positive manner, according to the template primer utilized, the rate of synthesis of the new DNA chain. Furthermore, the inhibition of DDDP activity observed at higher KA concentrations could be explained by hypothesizing the presence of a second binding-site for the compound.

Unlike HIV-1 RT, the DDDP activities of Mo-MuLV and AMV RT were inhibited irreversibly by KA. The fine analysis of this mechanism of inhibition, which is reminiscent of that observed for DNA polymerase α, Klenow fragment and several DNA and RNA polymerizing enzymes[24], is in progress.

Our data reveal that Kelletinins present a peculiar mechanism of inhibition compared to other non-nucleoside inhibitors of HIV-1 RT[10]. The inhibition of HTLV-1[23], AMV and Mo-MuLV RTs suggests that these compounds may be promiscuous against RT mutants and could circumvent, in combination therapy, the emergence of viral resistance observed under treatment with RT inhibitors[10,21]. Although KA is less potent against HIV-1 RT-RDDP activity than other non-nucleoside inhibitors, it inhibits DDDP activity (which is an alternative target for therapeutic intervention) with an efficacy comparable to these inhibitors[33,35]. Furthermore, it is interesting to mention that KA lacks significant cytotoxic effect in many cell culture and animal systems[23,36] at concentrations that inhibit DNA polymerase α [24] and RTs.

In conclusion, its effects on HTLV-1 replication, low cytotoxicity and polymerase active site-directed action suggest that KA is a potential antiviral compound in combination therapy and may be useful in the design of new structure-based reverse transcriptase inhibitors.

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