Kelletinin I and kelletinin A from the marine mollusc *Buccinulum corneum* are inhibitors of eukaryotic DNA polymerase α

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Received 8 March 1990; accepted 29 June 1990

Summary. The inhibitory effect of esters of p-hydroxybenzoic acid (kelletinins I and A), extracted from the marine gastropod *Buccinulum corneum*, have been tested on eukaryotic and prokaryotic enzymes of DNA metabolism such as DNA polymerases α and β, DNA polymerase I, Exo III, pancreatic DNAse I, micrococcal DNAse and *E. coli* RNA polymerase. Kelletinin I and kelletinin A inhibit preferentially DNA polymerase α. The inhibitory effect of kelletinin I involves the hydroxyl group of p-hydroxybenzoic acid.

Key words. Kelletinin I; kelletinin A; DNA polymerase α; inhibition of DNA replication.

Several esters of p-hydroxybenzoic acid have been reported to inhibit bacterial DNA and RNA synthesis [1]. It has been proposed that this inhibitory effect may be correlated with the antimicrobial activity of these compounds.

A p-hydroxybenzoate ester, kelletinin I [erythrityl tetrakis (p-hydroxybenzoate)], which inhibits the growth of *B. subtilis* and of L1210 leukemia cells, has been described in the marine gastropod *Kelletia kelletii* [2]. In addition to kelletinin I, a novel related compound [ribityl pentakis (p-hydroxybenzoate)] from *Buccinulum corneum* has been described in two papers and named kelletinin A [3] or buccinulin [4].

The present paper reports data indicating that kelletinin I inhibits preferentially eukaryotic DNA polymerase α, for example from *Xenopus* or mouse, while it inhibits only weakly mouse DNA polymerase β and *E. coli* DNA polymerase I and RNA polymerase. Exo III from *E. coli*, DNAse I from bovine pancreas and micrococcal DNAse from *S. aureus* are not affected. Kelletinin A shows an inhibitory effect like that of kelletinin I on DNA polymerase α activity. Further, we report data indicating the involvement of the hydroxyl group of the p-hydroxybenzoate moiety in the inhibition of DNA polymerase activities by kelletinin I.

Materials and methods

Kelletinin I and kelletinin A were isolated and purified as previously described [3]. The methoxy derivative of kelletinin I was prepared according to Tymiak et al. [2]. Glyceryl trikis (p-hydroxybenzoate) was obtained by dissolving p-hydroxybenzoic acid (0.5 g) in anhydrous glycerol (5 g) at about 60 °C. The mixture was then brought to 0 °C and gaseous HCl bubbled through it for 5 min. After 12 h at room temperature under mechanical stirring, the mixture was diluted with distilled water and the product extracted with diethyl ether. The extract (0.6 g) was chromatographed on a silica gel (Merck 70–230 mesh) column ($CHCl_3$) and three fractions of about 100 mg each were collected. The second fraction (Rf 0.7) was further purified on PLC Silica gel ($CHCl_3/CH_3OH$ 95/5) and the amorphous powder obtained was judged pure on the basis of chromatographic and spectral data. The NMR spectra were recorded on a 500 MHz-Bruker WM 500 in $CDCl_3$ ($CHCl_3$, δ 7.26); δ^1 = H-NMR: 7.97 (d, J = 8.7 Hz; 6H), 6.89 (d, J = 8.7 Hz; 6H), 6.61 (broad signal, −OH), 5.40 (tt, J = 5.5, 5.5 Hz; 1H), 3.87 (d, J = 5.5 Hz; 4H); MS m/z (%) 452 (M^+, 10%); U.V. λ max (CH_3OH) 260 nm, ε = 33800; I.R. γ max ($CHCl_3$) 3400–3200, 1728 cm^{-1}.

Pancreatic DNAse I, micrococcal DNAse, *E. coli* DNA polymerase I, RNA polymerase and Exo III were from Pharmacia; dATP, dCTP, dGTP, dTTP and ATP, CTP, GTP, UTP were from SIGMA; ^{3}H dTTP, ^{3}HUTP, ^{3}H dATP were from Amersham.

Xenopus laevis DNA polymerase α and mouse DNA polymerases α and β were assayed and purified as previously described [5–7]. *E. coli* DNA polymerase I was assayed in a mixture containing, in a total volume of 300 µl: 50 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 3.3 mM 2-mercaptoethanol, 80 µg gapped salmon sperm DNA, 0.165 mM each of dGTP, dCTP, dATP and 2.4 µCi of 0.4–0.8 µCi/nmole ^{3}H dTTP. After incubation at 37 °C the acid-insoluble material was processed as described [5]. *E. coli* RNA polymerase was assayed according to Burgess [8]. *E. coli* Exo III was assayed according to Regers et al. [9]. DNAse I was assayed in a mixture containing, in a total volume of 250 µl: 80 mM Tris-HCl (pH 7.4), 8 mM $MgCl_2$, 350 µg of native salmon sperm DNA, 200 µg of bovine serum albumin, 5–20 units of enzyme. After incubation at 37 °C the reaction was stopped by adding trichloroacetic acid (7% final concentration). After 15 min at 0 °C the samples were centrifuged at 35,000 × g for 20 min, and the OD^{260} of the supernatant determined. Micrococcal DNAse was assayed as described for DNAse I in a mixture containing, in a total volume of 250 µl: 80 mM Tris-HCl (pH 7.4), 5 mM NaCl, 350 µg of native salmon sperm DNA, 200 µg of bovine serum albumin and 5–20 units of enzyme.

Results

In table 1 the effect of kelletinin I (fig. 1, A) on several eukaryotic and prokaryotic enzymes of DNA metabolism and on *E. coli* RNA polymerase activity is shown. The drug is an inhibitor of DNA polymerase α activity from *X. laevis* and mouse. Mouse DNA polymerase β activity, *E. coli* DNA polymerase I and *E. coli* RNA polymerase activities are poorly inhibited compared to that of *Xenopus* or mouse DNA polymerase α. DNA nucleases such as Exo III, DNAse I and micrococcal DNAse activities are not inhibited, even at a concentration of 2×10^{-4} M.

The inhibitory effect of kelletinin I on DNA polymerase activities is not due to either of the two separate moieties of the molecule. In fact neither p-hydroxybenzoate nor erythritol inhibits *Xenopus* DNA polymerase α, nor does a mixture of the two (table 2).

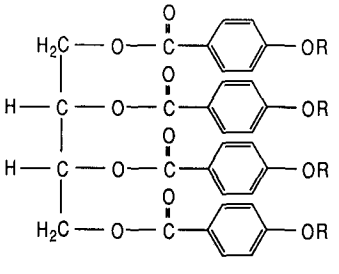
In table 3 the effects of kelletinin I-related compounds on DNA polymerase α activity from *X. laevis* are shown. Kelletinin A (fig. 1, C), isolated from *Buccinulum corneum*, shows an inhibitory effect comparable to that of kelletinin I, whereas the synthetic compounds, the methoxy derivative of kelletinin I (fig. 1, B) and glyceryl trikis (p-hydroxybenzoate) (fig. 1, D), do not affect the enzymatic activity.

In figure 2 the pH-dependence of kelletinin I inhibition of *E. coli* DNA polymerase I activity is depicted. *E. coli* DNA polymerase I was used, rather than DNA polymerase α, because the former enzyme exhibits a broad pH-range of activity. The inhibitory effect of kelletinin I is dependent on the pH of the reaction, reaching a maximum at about pH 7.6. A control experiment (data not shown) demonstrates that the lack of inhibitory effect is not due to hydrolysis of kelletinin I. A 1-h incubation at pH 9, under the DNA polymerase assay conditions, does not change the chromatographic properties of the molecule.

Table 1. Effect of kelletinin I on enzymes of DNA metabolism and on RNA polymerase

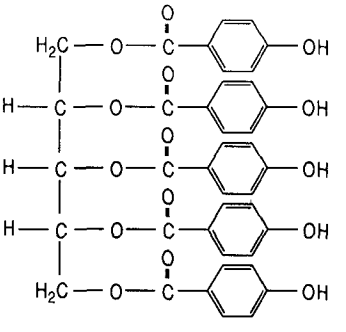
Enzyme	DNA	Kelletinin I	Residual activity
DNA polymerase α (*X. laevis*)	S.S. DNA gapped	1.0×10^{-5} M	48%
		2.5×10^{-5} M	11%
		5.0×10^{-5} M	1%
DNA polymerase α (mouse)	S.S. DNA gapped	2.5×10^{-5} M	9%
DNA polymerase β (mouse)	S.S. DNA gapped	1.0×10^{-4} M	49%
DNA polymerase (*E. coli*)	S.S. DNA gapped	2.5×10^{-5} M	100%
RNA polymerase (*E. coli*)	DNA native	2.5×10^{-5} M	85%
		5.0×10^{-5} M	50%
Exonuclease III (*E. coli*)	3'(^{3}H) dAMP DNA	2.0×10^{-4} M	100%
DNAse I (bovine pancreas)	DNA native	2.0×10^{-4} M	100%
DNAse micrococcal (*S. aureus*)	DNA native	2.0×10^{-4} M	100%

The residual activity was calculated after 6 min of incubation comparing the reaction in the presence of kelletinin I to a control assayed in conditions of linearity.

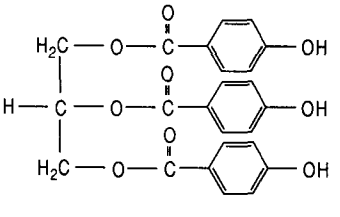


A R = H erythrityl tetrakis (p - hydroxybenzoate) (kelletinin I)

B R = CH_3 erythrityl tetrakis (p - methoxybenzoate)



C ribityl pentakis (p - hydroxybenzoate) (kelletinin A)



D glyceryl trikis (p - hydroxybenzoate)

Table 2. Effect of kelletinin I, p-hydroxybenzoate and erythritol on *Xenopus* DNA polymerase α activity

		^{3}H dAMP incorporated (pmole)	Residual activity
No addition		1160	100%
+ Kelletinin I	50 μM	4.3	1%
+ p-Hydroxybenzoate	500 μM	1140	98%
+ p-Hydroxybenzoate	2 mM	1150	99%
+ Erythritol	1 mM	1200	103%
+ Erythritol and p-hydroxybenzoate	200 μM 800 μM	1130	97%

2.4 units of enzyme for each assay were incubated for 10 min in a standard reaction mixture (see methods).

Table 3. Effect of kelletinin I-related compounds on *Xenopus* DNA polymerase α activity

		Residual activity
No addition		100%
Erythrityl tetrakis (p-hydroxybenzoate) (kelletinin I)	25 μM	15%
Ribityl pentakis (p-hydroxybenzoate) (kelletinin A)	25 μM	16%
Erythrityl tetrakis (p-methoxybenzoate) (methoxy kelletinin I)	25 μM	102%
Glyceryl trikis (p-hydroxybenzoate)	25 μM	98%

The assays were performed as described in the methods section using 2 units of *Xenopus laevis* DNA polymerase α. Residual activity was evaluated as in table 1.

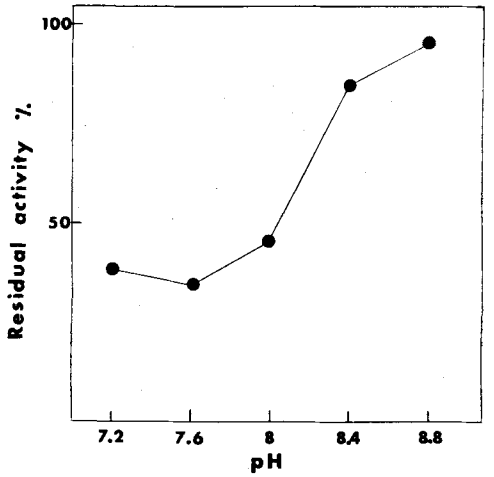


Figure 2. pH dependence of kelletinin I inhibition on *E. coli* DNA polymerase I. Residual activity was evaluated as in table 1.

Discussion

The present paper reports data indicating that kelletinin I and the related compound kelletinin A, isolated from the whelk *Buccinulum corneum*[3,4], are inhibitors preferentially of DNA polymerase α. In vitro experiments demonstrate that *Xenopus* or mouse DNA polymerase α is 5–10 fold more inhibited than mouse DNA polymerase β, or *E. coli* DNA polymerase I or RNA polymerase. Other enzymes of DNA metabolism such as Exo III, DNAse I, and micrococcal DNAse are not inhibited. The inhibitory activity of kelletinin I is considered to be associated with the hydroxyl group of p-hydroxybenzoate because of the following observations: a) the etherification of this p-hydroxyl group causes a loss in inhibitory activity against DNA polymerase α, and b) the inhibition is dependent on the pH of the reaction. At a pH value near to the pK_a of the p-hydroxyl group of benzoic acid (pK_a 9.2) DNA polymerase β (data not shown) and *E. coli* DNA polymerase I are not inhibited. However, the lack of inhibition at pH 8.8 could also be correlated with an enzymatic conformation less sensitive to kelletinin I at this pH.

The inhibitory effect of kelletinin I is strictly related to the structural peculiarities of the molecule. In fact, the two separate moieties of kelletinin I (erythritol and p-hydroxybenzoate) and the lower homolog of kelletinin I, glyceryl trikis (p-hydroxybenzoate), do not affect the DNA polymerase reaction.

Finally, the experiments reported above indicate that the inhibitory activity of these drugs is due to a specific interaction with the enzyme molecule rather than to a reaction with DNA, for the following reasons: a) Kelletinin I inhibits to various extents enzymes such as DNA polymerase α, DNA polymerase β, DNA polymerase I and RNA polymerase, which use DNA templates with essentially identical mechanisms. On the other hand, degradative DNA enzymes such as Exo III, DNAse I and micrococcal DNAse are not inhibited. b) Kinetic experiments with DNA polymerase α (data not shown) demonstrate that the enzyme is the limiting component of the reaction.

Acknowledgments. We thank Rosamaria Parisi for the outstanding technical assistance. Thanks are also extended to A. Crispino and A. Trabucco for the much-appreciated collection of *Buccinulum corneum*. This work was supported by grant No. 4206 33 'Ministero della Sanità – Istituto Superiore di Sanità – II° Progetto di Ricerca sull' AIDS'.

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0014-4754/91/010064-03$1.50 + 0.20/0