INFLUENCE OF TILORONE HYDROCHLORIDE ON THE SECONDARY STRUCTURE AND TEMPLATE ACTIVITY OF DNA

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1. Introduction

The dihydro-chloride salt of 2,7-bis(2-(diethyl-amino)ethoxy)-fluoren-9-one, referred to as tilorone hydrochloride (non-proprietary name) or bis-DEAE-fluorenone, is a broad spectrum antiviral compound [1] with antitumor activity [2,3]. Mayer and co-workers [4,5] have identified this compound as an interferon inducer and estabilished a relationship with the antiviral activity. Chandra et al. [6] have recently reported the inhibition of DNA polymerase activity in RNA tumor viruses by tilorone hydrochloride. This inhibition was found to be selectively dependent on the type of primer-template used in the enzymatic reaction. It was therefore interesting to study the mechanism of tilorone hydrochloride interaction to DNA, and its consequence on the template activity of DNA in DNA- and RNA-polymerase systems.

2. Materials and methods

Radioactively labeled compounds were obtained from NEN-Chemicals GmbH, Germany; other triphosphates and deoxytriphosphates were supplied by Boehringer Mannheim GmbH, Tutzing, Germany. Calf thymus DNA was isolated according to the procedure of Zamenhof [7]. DNase (2000 Kunitz units/mg protein) was obtained from Serva, Heidelberg. *E. coli* B and *E. coli* K-12 cells were supplied frozen (mid-log phase) by Miles Chemical Labs., Elkhart, USA. Tilorone hydrochloride was a gift of Merrel National Laboratories to Prof. C.L. Fox, Jr. All other chemicals were analytical grade reagents from Merck AG, Darmstadt.

RNA-polymerase reaction: RNA-polymerase was isolated from *E. coli* K-12 cells according to the procedure by Burgess [8] and kept in buffer containing 50% glycerol at −20°. The reaction mixture contained, in 0.25 ml, 0.04 M Tris, pH 7.9, 0.01 M $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.15 M KCl, 0.15 mM UTP, CTP and GTP, 0.15 mM ^{3}H-ATP and 0.15 mg per ml of calf thymus DNA. The reaction was started with 5–10 μg enzyme protein and incubations were carried out for 20 min at 37°. The reaction was stopped by adding 3 ml of 5% trichloroacetic acid (TCA) and serum albumin was used as carrier. The precipitate was collected on Whatman glass-fibre paper (GF/C) and washed 4 times with 3 ml of 2% TCA. The filter was dried and counted with toluol scintillation fluid in a Packard liquid scintillation spectrometer. Protein was estimated by the method of Lowry et al. [9].

DNA-polymerase reaction: DNA-polymerase was isolated from *E. coli* B cells according to the procedure of Richardson [10], and fraction VII obtained after DEAE-cellulose chromatography was used. The reaction was carried out in the presence of denatured DNA. The calf thymus DNA primed assay system contained (total vol 0.3 ml) 0.07 M glycine buffer, pH 9.2, 7 mM $MgCl_2$, 1 mM β-mercaptoethanol, 10 mμmoles each of dTTP, dCTP and dGTP, 2 μCi of ^{3}H-dATP, 20 μg of denatured calf thymus DNA. The reaction was started by adding 0.02 ml (approx. 50 μg protein) of the enzyme preparation. The complete mixture was incubated for 30 min at 37°, and

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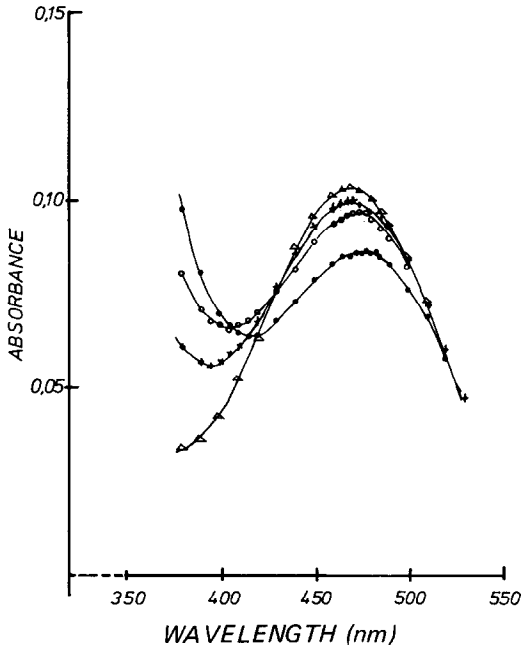

Fig. 1. Effect of calf thymus DNA on the visible absorption spectrum of tilorone hydrochloride. Samples contained 1×10^{-4} M of tilorone, 0.01 M Tris-HCl pH 7.0 and DNA at 0.5×10^{-3} M (+—+—+); 1×10^{-3} M (○——○——○); 2×10^{-3} M (●——●——●). No DNA was added to sample: (Δ——Δ——Δ).

the TCA-precipitable activity was measured on GF/C papers.

Physico-chemical measurements: Spectrophotometric measurements were carried out on a Zeiss PM QII or a Beckmann DU spectrometer.

3. Results and discussion

Interaction between nucleic acids and biologically active compounds may induce changes in the electronic spectra of the components. Tilorone hydrochloride in water shows two absorption maxima, in the ultraviolet region around 271 nm, and in the visible region around 470 nm. Thus the investigation of the long wavelength band, where DNA and RNA do not absorb, should provide some evidence whether or not the chromophore of tilorone hydrochloride is involved in the binding process. Fig. 1 depicts the absorption spectrum (350–500 nm) of tilorone hydrochloride alone (continuous line with triangles) or in the presence of various amounts of calf thymus DNA. There is a characteristic change in tilorone spectrum in the presence of DNA. In the presence of calf thymus DNA the visible absorption spectrum of tilorone hydrochloride is depressed and red shifted. This hypochromic effect of DNA on the absorption of tilorone chromophore is dependent on DNA concentration. The largest hypochromic effect is observed

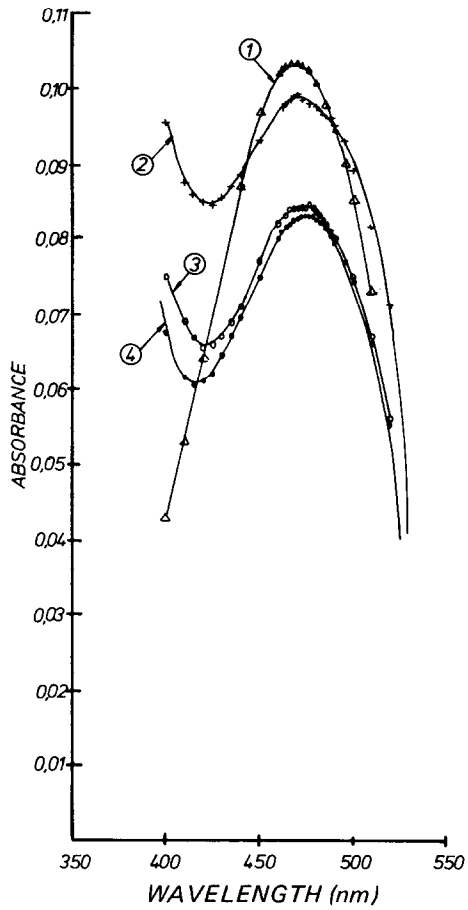

Fig. 2. Effect of native calf thymus DNA, denatured calf thymus DNA and yeast RNA on the visible absorption spectrum of tilorone in 0.01 M Tris-HCl pH 7.0. Curve 1 is the spectrum of free tilorone (1×10^{-4} M). Other curves depict the spectra of tilorone in the presence of yeast RNA (curve 2), denatured DNA (curve 3) and native DNA (curve 4). Molar concentrations of nucleic acids (2×10^{-3} M) refer to phosphorus content of the polymers.

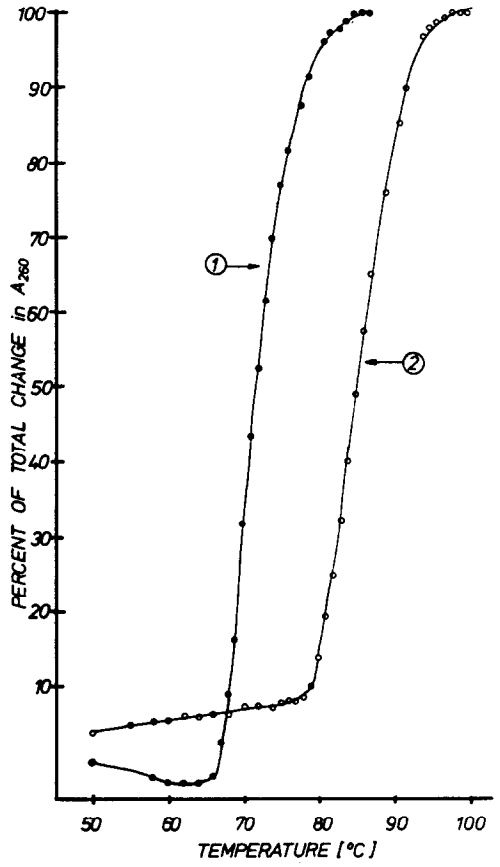

Fig. 3. Effect of tilorone on the thermal transition temperature (T_m) of calf thymus DNA. Solvent is 0.01 M Tris-HCl pH 7.0, and the DNA concentration is 5×10^{-5} M in all experiments. Curve 1 represents the melting profile of DNA in the absence of tilorone, and curve 2 is the melting profile of DNA in the presence of 2.5×10^{-6} M tilorone hydrochloride.

at 2×10^{-3} M DNA-P in a 1×10^{-4} M solution of tilorone hydrochloride.

The concentration-dependent effect of calf thymus DNA on the visible absorption spectrum of tilorone hydrochloride indicates that the tilorone chromophore interacts with DNA. Fig. 2 depicts the visible absorption spectra of tilorone alone (curve 1), or in the presence of yeast RNA (curve 2), denatured DNA (curve 3) and native double-stranded DNA (curve 4). The visible spectra indicate that at equimolar concentrations, DNA in its double-helical state produces largest changes in the absorption spectrum of tilorone, whereas the effect of single-standed DNA is slightly weaker. In contrast, the yeast RNA exerts only a slight effect on the visible spectrum of tilorone hydrochloride. These data indicate a specificity of the tilorone chromophore towards DNA.

Further information on the binding of tilorone with DNA was derived by studying the thermal melting of the complex. In order to characterize the stability of DNA secondary structure in the presence of tilorone, temperature profiles were run at tilorone/DNA-P molar ratio of 1:20 (fig. 3). Tilorone hydrochloride shows a large increase in the thermal transition temperature (T_m) of native DNA; the T_m of calf thymus DNA was raised from 71.6 to 85.2° under these conditions.

The interaction of tilorone hydrochloride to native as well as denatured DNA encouraged us to study the template activity of the complaxes in DNA- and RNA-polymerase systems from *E. coli*. Table 1 shows the effect of tilorone on the priming activity of denatured DNA in DNA-polymerase reaction. As follows from the results there is a concentration dependent inhibition of ^{3}H-dAMP incorporation into DNA by tilorone hydrochloride. Concentration as low as 5 μg per reaction mixture inhibits more than 80% the incorporation of ^{3}H-dAMP into DNA. At 15 μg/reaction mixture the reaction is completely blocked by tilorone.

Table 1
Inhibition of DNA-dependent DNA polymerase reaction (*E. coli* B) by tilorone hydrochloride.

System	Tilorone hydrochloride concentration (μg/reaction mixt.)*	^{3}H-dAMP incorporation into DNA (cpm/reaction mixt.)	% of Control
Without DNA	—	67	0.2
Without enzyme	—	35	0.1
Complete	None	29,087	100
	5	5,395	18.3
	10	1,380	4.7
	15	372	1.2
	20	166	0.57

* μg/0.30 ml of reaction mixture. The reaction conditions are described under Materials and methods.

Table 2
Inhibition of DNA-dependent RNA polymerase reaction (*E. coli* K-12) by tilorone hydrochloride.

System	Tilorone hydrochloride concentration (μg/reaction mixt.)*	^{14}C-AMP incorporation into RNA (cpm/reaction mixt.)	% of Control
Without UTP,CTP and GTP	–	14	2.1
Without DNA	–	11	1.6
Complete	None	652	100
	25	471	72
	50	313	46
	100	201	31

* μg/0.25 ml of reaction mixture. The reaction conditions are described under Materials and methods.

The inhibiting activity of tilorone hydrochloride on the DNA-dependent RNA-polymerase reaction is shown in table 2. Compared to the DNA-polymerase reaction, the RNA-polymerase reaction requires large amounts of tilorone hydrochloride for its inhibition; no significant inhibition was observed below 15 μg/reaction mixture of tilorone hydrochloride. Whereas this amount of tilorone was able to completely inhibit the DNA polymerase reaction (see table 1). One explanation is that in the RNA-polymerase reaction the DNA concentration is approx. 2.5 times more than that used in the DNA-polymerase reaction. However, this may not be the only reason for such differences. Our preliminary spectrophotometric data show that Mg^{2+}ions influence the tilorone binding to DNA. Since the Mg^{2+}ion concentrations in both systems are different, this may account for the variable sensitivity of both systems towards tilorone hydrochloride.

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