MODE OF TILORONE HYDROCHLORIDE INTERACTION TO DNA AND POLYDEOXYRIBONUCLEOTIDES

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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 [2–4]. Mayer and co-workers [5, 6] have identified this compound as an interferon inducer and established a relationship with the antiviral activity. However, recently a lack of correlation between interferon induction and viral protection by tilorone hydrochloride has been reported [7].

Tilorone hydrochloride has been found to form a molecular complex with DNA and to inhibit the DNA template functions in DNA- and RNA-polymerase reactions *in vitro* [8]. It was also reported to inhibit DNA polymerase activity in RNA tumor viruses [9]. The drug appears to belong, therefore, to that class of compounds which exert a variety of biological effects by engaging in complex formation with DNA. This paper describes some specific parameters involved in tilorone hydrochloride interaction with DNA and polydeoxyribonucleotides.

2. Materials and methods

Tilorone hydrochloride was a gift of Merrel National Laboratories to Dr. C.L. Fox. DNA from *Cl. perfringens* was obtained from Calbiochem, Luzern; polydeoxyribonucleotides were obtained from Miles Laboratories Inc. and P.-L Biochemicals Inc., USA.

Calf thymus DNA was isolated according to the method of Zamenhof [10]. Some samples were further purified by pronase digestion and phenol extraction. Bacterial DNA was isolated according to the procedure described by Thomas et al. [11]. Apurinic and apyrimidinic DNA were prepared from calf thymus DNA by the methods of Tamm et al. [12] and Shapiro [13] respectively. Sonicated DNA was prepared as reported by Doty et al. [14].

[9-^{14}C]Tilorone hydrochloride (600 000 cpm/mg) was synthesized from biphenyl-2-carboxylic-[^{14}C]-acid, as described elsewhere [15]. Solutions containing DNA and labeled tilorone (with or without magnesium ions) were layered on 5–20% linear sucrose gradients and centrifuged in a SW 39 rotor for 18 hr at 0°. Fractions (8 drops each) were collected by puncturing the bottom, diluting with water and the radioactivity measured using dioxane scintillation fluid.

Unless otherwise indicated, all binding experiments were performed in 0.01 M Tris-HCl buffer (pH 7.0). Thermal denaturation studies were carried out with a Zeiss PMQ II spectrophotometer. Viscosities were

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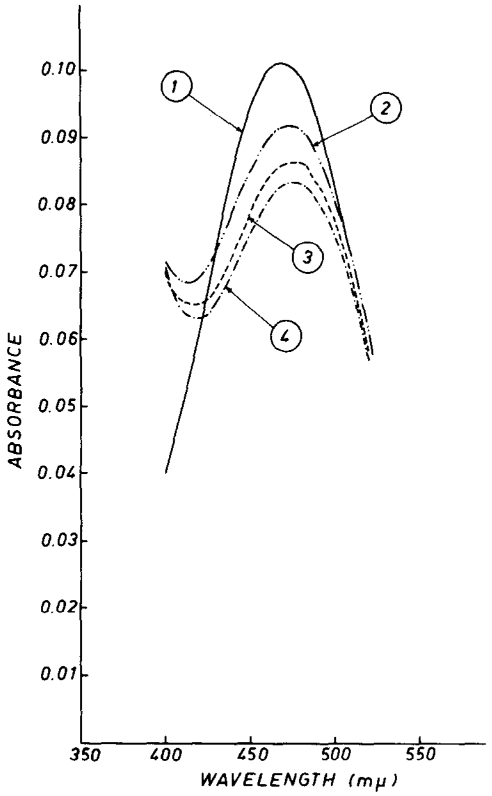

Fig. 1. Effect of Na^+ and Mg^{2+} on the visible absorption spectrum of the tilorone–DNA complex. Samples contained 10^{-4} M tilorone, 4×10^{-3} M DNA-P, 0.01 M Tris-HCl (pH 7.0) and 0.01 M $MgCl_2$ (curve 2) or 0.1 M NaCl (curve 3). Curve 1 is the spectrum of free tilorone; curve 4 is the spectrum of the tilorone–DNA complex in the absence of Na^+ and Mg^{2+}.

measured at 20° by means of a Zimm-Crother low shear viscosimeter adjusted to give a shear stress of less than 0.002 dynes/cm^2. Intrinsic viscosities pertaining to drug–DNA complexes in various states "r" were obtained by linear extrapolation of the reduced viscosity measured at different DNA concentrations. Dilutions of DNA–drug mixtures were carried out at constant "*r*" by using a drug solution known to be in dialysis equilibrium with the complex in state "*r*". Sedimentation analysis were carried out in a Beckman Model E ultracentrifuge.

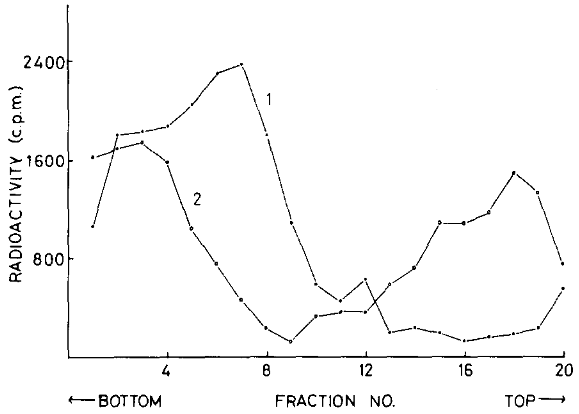

Fig. 2. Effect of Mg^{2+} on the binding of [9-^{14}C] tilorone-hydrochloride to salmon sperm DNA. Samples containing 2×10^{-4} M [9-^{14}C]tilorone-hydrochloride and 15×10^{-3} M DNA-P (curve 1) with 0.01 M $MgCl_2$ (curve 2) were layered on a linear sucrose gradient (5–20%) and centrifuged in SW 39 rotor for 18 hr. Experimental details are described in text

3. Results and discussion

In the presence of calf thymus DNA the visible absorption spectrum of tilorone hydrochloride is depressed and red shifted [8]. This hypochromic effect of DNA on the absorption of tilorone hydrochloride chromophore is dependent on DNA concentration. In the present study we have found that the hypochromic effect of native DNA on the absorption of tilorone chromophore is partially reversible by Na^+ and Mg^{2+} ions. Fig. 1 depicts the absorption spectra (350–550 nm) of tilorone hydrochloride alone, 1×10^{-4} M (curve 1), in the presence of 4×10^{-3} M DNA-P (curve 4) containing 0.01 M $MgCl_2$ (curve 2) or 0.1 M NaCl (curve 3). It follows from these results that the DNA–drug interaction is very sensitive to magnesium ions.

The effect of magnesium ions on tilorone binding to DNA was confirmed by density-gradient studies using labeled tilorone hydrochloride. As follows from fig. 2 the radioactivity bound to DNA is strongly effected by magnesium ions (curve 2). The Mg^{2+} ion

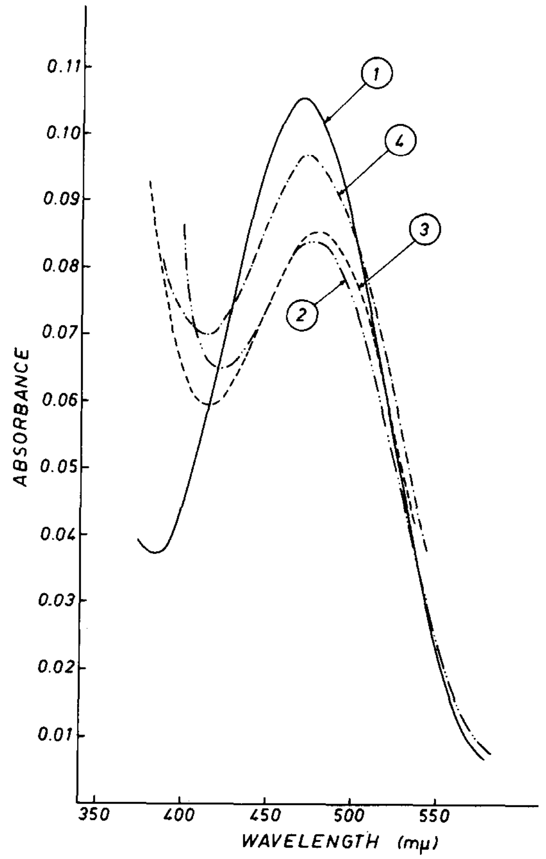

Fig. 3. Effect of apurinic and apyrimidinic DNA on the visible absorption spectrum of tilorone in 0.01 M Tris-HCl (pH 7.0). Curve 1 is the spectrum of free tilorone (10^{-4} M); other curves are the spectra of tilorone in the presence of native calf thymus DNA (curve 2), apyrimidinic DNA (curve 3) and apurinic DNA (curve 4). Molar concentrations of nucleic acids as phosphorus (2×10^{-3} M) content of the polymers.

concentration in this experiment was the same as under fig. 1. These studies indicate that electrostatic forces contribute greatly to the binding process. The interaction between tilorone and DNA may, however, involve other kinds of forces. Tilorone forms a reversible complex with DNA, since the drug could be completely dissociated from a DNA-cellulose column. Interaction of apurinic and apyrimidinic DNA's with tilorone hydrochloride also gave spectral changes, as

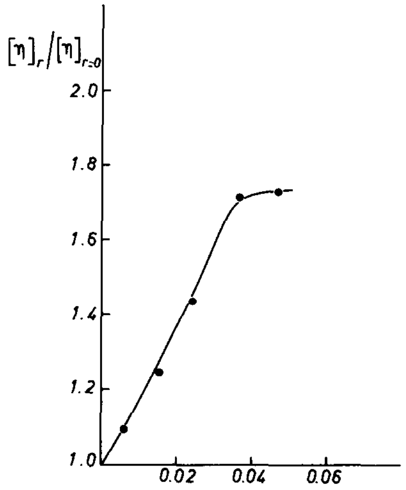

Fig. 4. Intrinsic viscosity of tilorone–DNA complex relative to intrinsic viscosity of DNA alone. Intrinsic viscosities were determined as described under Materials and methods. Conditions of viscosity measurements: 20°, 0.01 M Tris-HCl (pH 7.0).

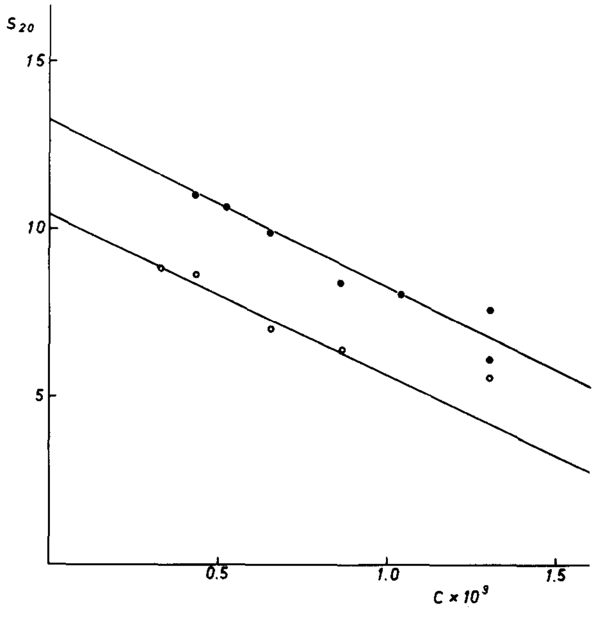

Fig. 5. Effect of tilorone on the sedimentation coefficient of DNA. C is the molar concentration of DNA phosphorus. Filled points apply to DNA alone; open circles to tilorone–DNA complex at a molar ratio of drug to DNA-P of 0.1.

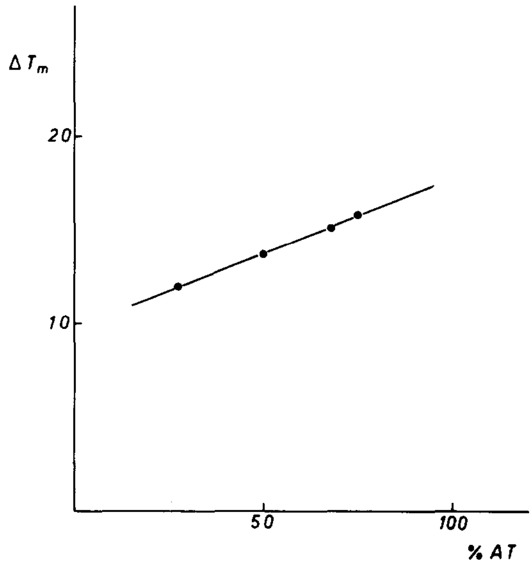

Fig. 6. Effect of tilorone on the T_m with DNA preparations from various sources of different base compositions. T_m values were obtained at a molar ratio of drug to DNA-P of 0.05. The T_m of DNA (5×10^{-5} M) with and without tilorone was determined in a medium of 0.01 M Tris-HCl (pH 7.0). AT-content of *B. cereus* DNA (65%), calf thymus DNA (58%), *E. coli* DNA (50%) and *M. lysodeikticus* DNA (28%); these DNA samples were used in these studies.

shown in fig. 3. However, only with the apyrimidinic DNA, the spectrum of the bound drug was similar to that found with native DNA.

The absorption spectrum studies presented above merely reflect the electronic environment of the molecule and do not give specific information about the type of interaction. The data which must be accounted for in considering a physical mode for the binding process can be derived from several different approaches. Hydrodynamic measurements on the DNA–drug complex are of interest, since Lerman [16] has established that an increase in the intrinsic viscosity of DNA and a decrease in the sedimentation coefficient of the polymer are two criteria for intercalation of ring systems between base pairs of a double-helical DNA.

Fig. 4 shows the relationship between the intrinsic viscosity of DNA and the amount ("*r*") of bound tilorone. The intrinsic viscosity of the complex increases with *r* up to a limiting value of about 0.05. The maximum relative enhancement of viscosity was about 1.7. In addition, at the same ionic strength and at a ligand to DNA-P molar ratio of 0.1, the sedimentation rate of DNA was decreased to 78% of the value in the absence of ligand (fig. 5).

These observations are consistent with an intercalative mode of binding in the interaction of tilorone hydrochloride with double-helical DNA. These results were not examined in attempt to verify whether they agree with measurements of the length increase on sonicated DNA. For this reason, the intercalation model of the DNA complex remains tentative.

The interaction of tilorone hydrochloride with native DNA stabilizes the double helical structure of the macromolecule towards thermal denaturation [8]. The effect of tilorone hydrochloride on the thermal denaturation of DNA's from various sources having different base composition is shown in fig. 6.

At a drug to DNA-P molar ratio of 0.05, the ΔT_m increased with increasing AT content of the DNA. This observation indicates that tilorone hydrochloride preferentially binds to the dAT portions of the DNA molecule. This hypothesis is confirmed by the strong effect of tilorone hydrochloride on the thermal transition temperature of poly d(A–T), $\Delta T_m = 29°$.

An intercalative mechanism for binding of a ligand to DNA is consistent with a stabilization of the double helix. Such a stabilization, however, does not constitute proof of intercalation. But, when considered with the evidence of the results reported in this paper, showing increased viscosities and decreased sedimentation rate of DNA, one may conclude that the large increase of T_m points to an intercalative mode of binding.

Acknowledgement

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References

[1] R.F. Krueger and S. Yoshimura, Federation Proc. 29 (1970) 635.
[2] R.H. Adamson, J. Natl. Cancer Inst. 46 (1971) 431.
[3] A.E. Munson, J.A. Munson and W. Regelson, Intl. Colloq. on Interferone Interferone Inducers, Leuven, Belgium, Abstract No. 27 (1971).

[4] A.E. Munson, J.A. Munson, W. Regelson and G.L. Wampler, Cancer Res. 32 (1972) 1397.
[5] G.D. Mayer and R.F. Krueger, Science 169 (1970) 1214.
[6] G.D. Mayer and B.A. Fink, Federation Proc. 29 (1970) 635.
[7] D.J. Giron, J.P. Schmidt and F.F. Pindak, Antimicrobial Agents and Chemther. 1 (1972) 78.
[8] P. Chandra, F. Zunino, A. Zaccara, A. Wacker and A. Götz, FEBS Letters 23 (1972) 145.
[9] P. Chandra, F. Zunino and A. Götz, FEBS Letters 22 (1972) 161.
[10] S. Zamenhof, Biochemical Preparations 6 (1958) 8.
[11] C.A. Thomas, K.I. Berns and T.J. Kelly, in: Procedures in Nucleic Acid Res., eds. G.L. Cantoni and D.R. Davies (Harper Int. Ed., New York, 1967) p. 535.
[12] C. Tamm, M.E. Hodes and E. Chargaff, J. Biol. Chem. 195 (1962) 49.
[13] H.S. Shapiro, Methods in Enzymology 12A (1967) 212.