COMPLEXES OF DNA WITH INTERFERON-INDUCING FLUORENE AND FLUORENONE DERIVATIVES

Kh. A. Khalmuratov, A. P. Zlatkovskii, S. A. Auelbekov, B. V. Tyaglov, V. I. Permogorov, and Kh. A. Aslanov

UDC 615.281.8

Information is given on the influence of fluorene and fluorenone derivatives on the melting point of thymus DNA and their interferon-inducing activity. The substances investigated form complexes predominantly with the AT pairs of DNA. A comparative electro-phoretic study of complexes of the substances investigated and of ethidium bromide has shown that the fluorene and fluorenone derivatives do not intercalate into the DNA double helix. The change in the melting point of DNA correlates with the chemical structure of the compounds investigated and depends on the volume of the substituent on the nitrogen atom of the side chain.

At the present time, interferon has entered the clinical practice of the treatment of various diseases [1-3]. However, there is no weakening of interest in the use of interferon inductors for the prophylaxis and treatment of viral and oncological diseases [4]. One of the main disadvantages of interferon inductors is the development of hyperreactivity to a repeated induction of interferon — a phenomenon regularly developing in response to the administration of one and the same drug that is overcome by the alternating administration of inductors of different natures [5].

One interferon inductor that is active on peroral administration and has been fairly well studied is tilorone hydrochloride — 2,7-bis(2-ethylaminoethoxy)fluorenon-9-one hydrochloride — which induces high titers of interferon in rodents [6]. Interaction into the nucleic acids of the cell has been suggested as a possible mechanism of interferon-ogenesis responsible for the action of tilorone [7, 8].

We have investgated the complexes of a group of fluorene and fluorenone derivatives with DNA. These compounds were also studied for their capacity for inducing interferon in mice.

2,7-Bis(piperidinoacetylamino)fluoren-9-one (I), 2,7-bis(cytisinoacetylamino)fluoren-9-one (II), 2,7-bis(morpholinoacetylamino)fluoren-9-one (III), 2,7-bis(decahydroquinolinoacetylamino)fluoren-9-one (IV), 2,7-bis(anabasinoacetylamino)fluoren-9-one (V), 2,7-bis(diethylaminoacetylamino)fluoren-9-one (VI), 2,7-bis(dibutylaminoacetylamino)fluoren-9-one (VII), 2,7-bis(salsolinoacetylamino)fluoren-9-one (VIII), 2,7-bis(diphenylaminoacetylamino)fluoren-9-one (IX), 2,7-bis(piperidinoacetylamino)fluorene (X), 2,7-bis(salsolinoacetylamino)fluorene (XI), and 2,7-bis(2-ethylaminoethoxy)fluoren-9-one, or tilorone, were synthesized.

Table 1 gives results for the influence of the fluorene and fluorenone derivatives on the melting point of thymus DNA and their interferon-inducing activities. Practically all the fluorene and fluorenone derivatives studied caused the stabilization of double-stranded DNA, the stabilizing effect being expressed more strongly for compounds (I, V, VI, and X) (at the same DNA/ligand ratio), probably because of the absence from these compounds of voluminous substituents in the molecule at the quaternary nitrogen atoms, which possibly create steric hindrance in the binding of ligands. The appearance in the side chain of voluminous substituents having a hydrophobic nature (compounds (II) and (IX)) diminishes their effect on the thermal stability of the DNA molecules.

The interferon-inducing activities of the substances investigated depend both on their chemical structure and on the dose used. Fluorenone derivatives having voluminous groups in the lateral moiety induce interferon with low activity.

V. I. Lenin Tashkent State University. Translated from Khimiya Prirodnykh Soedinenii, No. 3, pp. 404-409, May-June, 1988. Original article submitted September 25, 1987; revision submitted January 25, 1988.

0009-3130/88/2403-0343 $12.50

TABLE 1. Influence of Fluorene and Fluorenone Derivatives on the Melting Point of Thymus DNA and Their Interferon-inducing Activities

Compound	DNA/ligand ratio, molar	mp, °C	Dose of drug, mg/kg	Interferon activity, IU_{50}/ml
I	10:1	16,5	25	20
	3:1	36,5	100	64
II	10:1	5,4	100	16
III	10:1	4,9	100	20
IV	10:1	5,6	100	10
V	10:1	5,7	25	80
	3:1	10,4	100	640
VI	10:1	15,4	25	64
	3:1	29,2	100	160
VII	10:1	5,0	100	16
VIII	10:1	5,9	100	32
IX	10:1	5,3	100	10
X	10:1	6,3	100	10
XI	10:1	3,1	100	10
Tilorone	3:1	36,4	100	320
	10:1	23,3	25	80

In a dose of 25 mg/kg, compound (V) induced serum interferon with an activity of 80 IU_{50}/ml, and an increase in the dose was accompanied by a rise in the activity of the induced interferon to 640 IU_{50}/ml. We have shown that tilorone hydrochloride in a dose of 25 mg/kg induces interferon with an activity of 80 IU_{50}/ml, and in a dose of 100 mg/kg one of 320 IU_{50}/ml. There is inadequate information in the literature on the activity of induced interferon. Thus, 18 h after the peroral administration of 250 mg/kg of tilorone the activity of serum interferon reached 5000 units/ml [10, 11]. According to other authors, on peroral administration in the same dose the drug induced interferon with activities of 750 and 320 IU_{50}/ml [12]. The activity of tilorone-induced interferon in mice depends on the dose of the drug, on the number of doses, on the addition of high-molecular-weight components (polysaccharides), and on the line of mice. It follows from the results obtained on the interferon-inducing activity of the compounds synthesized that, among the substances studied, compound (V), containing an anabasine fragment in the side chain, induced the highest titer of interferon.

It follows from Table 1 that the correlation between the change in $T_m - \Delta T -$ of the polynucleotide as the result of the formation of complexes with the compounds under investigation and their capacities for inducing interferon is expressed feebly.

Thus, all the fluorene and fluorenone derivatives considered in the present paper are capable of binding with DNA and raising T_m, ΔT for the various compounds differing substantially. One of the reasons for this may be the capacity of these substances for binding with DNA through various types of interactions. This question is of fundamental interest [13]. Thus, tilorone is an inductor of interferon, has a considerable effect on the thermal stability of DNA, and, according to the literature, intercalates into the DNA double helix [8].

To answer these questions, we investigated the dependence of ΔT on the logarithm of the ionic strength of the solution. It is known that if a complex is formed as the result of various types of interactions between the ligands and the polynucleotide matrix the dependence of ΔT on the logarithm of the ionic strength may reveal this fact [14].

Figure 1 shows curves of the dependence of ΔT on the logarithm of the ionic strength of the solution. With an increase in the ionic strength of the solution ΔT falls. It follows from Fig. 1 that the course of the dependence of ΔT on the ionic strength is the same for the compounds investigated (curves 2 and 3 fall on curve 1 when multiplied by the factors 1.6 and 4.5, respectively), and the rapid fall in ΔT indicates that in these cases we are dealing with an ionic interaction. However, when there is an intercalation of ligands into DNA the dependence of the parameters of the binding of ligands with the DNA on the ionic strength differs from the curves that we have observed [14].

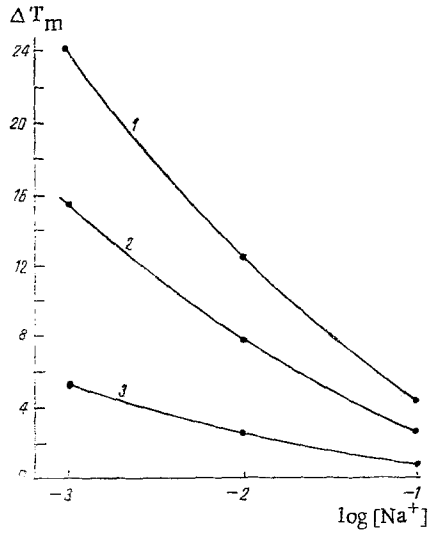

Fig. 1. Dependence of the melting points of complexes of tilorone (curve 1), compound (VI) (curve 2), and compound (VII) (curve 3) with calf thymus DNA on the logarithm of the ionic strength of the solution. Concentration of DNA 7.5 μg/ml. Ratio 2 D/R [sic] — $0.1 \cdot 10^{-3}$ M Tris-HCl buffer, pH 7.0, containing $5 \cdot 10^{-5}$ M EDTA.

To answer the question of the intercalation of DNA into the compounds studied, we investigated the binding of the ligands with circularly closed plasmid DNAs. It had been established previously that, on being bound to circular DNA, ligands capable of intercalating into the helix change the number of supertwists of this DNA, whereupon, for example, the mobilities of these complexes on gel electrophoresis change from that of the supercoiled DNA without a ligand. On the formation of complexes of fluorene and fluorenone derivatives with supercoiled plasmid DNA its mobility in electrophoresis in agarose gel scarcely changed in contrast to the plasmid DNA treated with ethidium bromide, for which the mobility of the complex decreased. However, these experiments cannot be treated unambiguously. The binding constants of the ligands studied were lower than those for ethidium bromide (by approximately two orders of magnitude) [8], and in an electric field these compounds may dissociate and the pattern will be blurred. In view of this fact, we studied complexes of the compounds under investigation with circular DNAs by the following scheme. Supercoiled plasmid DNA was treated with restrictase EcoRI, for which there is one restriction site in the PLA plasmid used; to the linear DNA so obtained the ligand was added in a concentration sufficient to bind all the supertwists in the supercoiled plasmid DNA. After this operation, the DNA (in the presence of the ligands) was ligated, which led to open circular DNA molecules with the bound ligands. The ligands, and also the ligase, were washed out from the DNA with n-butanol. If, in this procedure, the ligand was intercalated into the DNA, the DNA changed the number of its twists, which led to a change in the number of supertwists in it, as well, because of which the mobilities of such DNAs became close to that which was present in the plasmid initially. If the ligands are not intercalated, the mobility changes insignificantly. An electrophoretic study of complexes of DNA with the ligands under investigation — including tilorone — showed that they do not intercalate into the DNA double helix.

Using compounds (VI) and (VII) as examples, we studied the influence of nucleotide composition on the capacity for stabilization of the double-stranded structure of DNA (Table 2). It follows from the results in Table 2 that ΔT rises with an increase in the amount of AT pairs. Fluorene and fluorenone derivatives have an affinity for AT pairs. Muller and Crothers [15], using a large number of compounds, showed that ligands capable of intercalating have affinity for GC pairs and, conversely, those not capable of intercalating have an affinity for AT pairs. Fluorene and fluorenone derivatives have an affinity for AT pairs and, as was shown above, do not intercalate into DNA, this being in complete harmony with Muller and Crothers' scheme.

TABLE 2. Influence of Fluorene Derivatives on the Melting Point of Polydeoxyribonucleotides with Different Amounts of GC Pairs

Polydeoxyribonucleotide	Amount of GC pairs, %	mp of the free polydeoxyribonucleotide, °C	mp, °C	
			VI	VII
1. Poly[d(A–T)]	0	27,7	17,4	6,4
2. Phage T4 DNA	35,5	49.3	16.5	5,4
3. Phage T4 (mutant) DNA	35,5	48,0	16,3	5,6
4. DNA from Methanolobus tindarius	41,5	53,4	15,5	5,0
5. Poly[d(G–C)], poly[d(T–A)]	50	58,1	15,1	4,8
6. DNA from Comlobacter vibroides	61,3	71,0	14,7	4,4
7. DNA from Strept. coelicolor	70.9	71,0	14,7	4,4
8. Poly[d(G–C)]	100	83,4	13,6	3,6

An important characteristic of complexes is the localization of the ligand in the DNA matrix, and we therefore studied complexes of compounds (VI) and (VII) with phage T4 DNA and the DNA of the amber mutant of T4 in relation to β-glucosyltransferase. These DNAs differ from one another by the fact that the phage T4 DNA is glycosylated completely at the 5-hydroxymethylcytosine residues, while the DNA of the mutant phage was not glycosylated. The glucose residues are localized in the large groove [16] and create steric hindrance for the binding of ligands addressed to the large groove of the double helix. It follows from Table 2 that the two DNAs mentioned were stabilized by the ligands similarly, which indicates that in the process of complex-formation compounds (VI) and (VII) are localized in the small groove of the DNA double helix.

EXPERIMENTAL

The interferon-inducing activity of the fluorene and fluorenone derivatives was studied on random-bred white mice weighing 10-12 g by the intraperitoneal injection of aqueous solution of the compounds in a volume of 0.1 ml. The mice were sacrificed 24 h after the injection of the substances and blood was collected from the subclavian artery. The blood from the mice of each group was combined, kept for 30 min at 37°C, and then centrifuged at 1000 rpm for 10 min. The clarified mouse blood serum was stored at +4°C. Interferon activity in the mouse serum was determined by titration in a monolayer of L_{929} cells grown on the bottom of test-tubes or by a micro method in 96-well polystyrene panels. The titers of the interferon induced in the blood of the animals were determined against the virus of vesicular stomatitis. As the unit of interferon activity we took the reciprocal of the dilution of the serum that caused 50% protection of the cells from the cytopathic action of 100 TCD_{50} of the test virus.

Plasmid PLA-100, with a size of 9.1 kb was treated under standard conditions with restrictase EcoRI. Then the restrictase was eliminated by heating to 65°C, the ligand was added in a ratio of 700:1 and, without being freed from the restriction buffer, it was ligated at 12°C for 12 h. The ligands and the ligase were extracted with normal butanol, and the plasmid was precipitated with ethanol and was subjected to electrophoresis for 2 h.

Gel Electrophoresis. For this we used 0.8% agarose gel and standard Tris-acetate buffer, pH 7.5, at a voltage of 12 V/cm.

Restriction and ligation were carried out in a buffer having pH 8 and containing 33 mM Tris base, 66 mM potassium acetate, 10 mM magnesium acetate, and 100 mM sodium chloride. All the reagents used were of kh. ch ["chemically pure"] or os.ch. ["ultrapure"] grade.

Polynucleotides. We used the synthetic polynucleotides poly[d(A)], poly[d(T)], poly[d(G–C)], and poly[d(A–T)] (FRG) and commerical preparations of DNA isolated from calf thymus (USA). Preparations of DNA from bacteriophage T4 and also from Comlobacter vibrodies and Methanolobus tindarius were isolated by Marmur's method [17], with subsequent purification in a cesium chloride gradient. A DNA preparation isolated from Strept. coelicolor (2) was provided by I. A. Sladkova (All-Union Scientific-Research Institute of the Genetics and Selection of Industrial Microorganisms of Minmedmikrobioprom SSSR).

Before the experiments all the polynucleotides were subjected for 24 h to dialysis against 0.001 M Tris-HCl buffer containing 0.001 M NaCl and $5 \cdot 10^{-5}$ M EDTA, pH 7.0. After dialysis the preparations were stored in quartz or Teflon vessels. Buffer solutions were prepared from distilled water with a specific resistance of $2 \cdot 10^5$ Ω/cm.

Heat Denaturation. Immediately before the experiments, the fluorene and fluorenone derivatives were mixed in the above-mentioned buffer. The change in the optical density of the DNAs and polynucleotides were recorded on an Acta—MIV spectrophotometer at 260 nm. Heating was performed in a thermostated cell at the rate of 0.25 deg/min. The temperature of the sample was measured in the cell by means of a Pye-Unicam 373 thermocouple with an accuracy of ± 0.1°C; the melting points of the complexes were determined at the point corresponding to half the maximum hyperchromic effect. Since the optical density of the ligands does not change in the 15—98°C interval, the hyperchromic effect was calculated from the following formula:

$$h,\ \% = \frac{A_m - A_i}{A_i} \cdot 100\%,$$

where A_i and A_m are the densities of solutions of the complexes of the polynucletotides with the fluorene and fluorenone derivatives at the initial temperature and at the melting point, respectively. The intrinsic absorption at 260 nm of the ligands studied was deducted. To obtain each melting curve, 3—5 independent measurements were made. Melting points were determined with an accuracy of ± 0.2°C.

SUMMARY

Fluorene and florenone derivatives possessing interferon-inducing activity exert an influence on the melting point of DNA, and the change in the melting point correlates with chemical structure and depends on the volume of the substituent of the nitrogen atom of the side chain.

Fluorene and fluorenone derivatives do not intercalate into the DNA double helix, and complex-formation takes place mainly with AT pairs, the localization of the compounds studied being in the small groove of the DNA. The change in the melting point on the formation of complexes by these compounds correlates feebly with their capacity for inducing interferon.

LITERATURE CITED

1. Yu. T. Kalinin, A. A. Vorob'ev, V. V. Parfenov, et al., Zh. Mikrobiol. Epidemiol. Immunobiol., 10, 3 (1984).
2. A. Billian, Adv. Virus Res., 1, 131 (1985).
3. F. G. Hayden, S. E. Mils, and M. E. Sohns, J. Infect Dis., 148, No. 5, 914 (1983).
4. F. I. Ershov, S. A. Auelbekov, and Kh. A. Alsanov, The Molecular Biology of Viruses [in Russian], Moscow (1985), p. 209.
5. F. I. Ershov and V. I. Zhdanov, Interferon Inducers [in Russian], Moscow (1982), p. 7.
6. G. D. Mayer, Pharm. Ther., 8, 173 (1980).
7. A. S. Sadykov, F. I. Ershov, A. S. Novokhatskii, et al., Interferon Inducers [in Russian], Fan, Tashkent (1978), p. 198.
8. P. Chandra and M. Woltersdorf, FEBS Lett., 41, 643 (1974).
9. S. A. Auelbekov, Kh. A. Khalmuratov, and Kh. A. Aslanov, Synthesis and Biological Activity of Organic Compounds: A Collection of Papers [in Russian], Tashkent State University (1985), p. 49.
10. E. D. Clerg, Antibiot. Chemother., 27, 251 (1980).
11. D. A. Stringfellow, Antimicrob. Agents Chemother., 11, 984 (1977).
12. W. Witkowski, S. Hoffmann, and A. Veckenstedt, Acta Biol. Med. Germ., 38, 733 (1979).
13. A. G. Karapetyan, V. I. Permogorov, M. D. Frank-Kamenitskii, and Yu. S. Lazurkin, Mol. Biol., 6, 867 (1972).
14. V. I. Permogorov, Yu. S. Lazurkin, and S. Z. Shmurak, Dokl. Akad. Nauk SSSR, 155, 1440 (1964).
15. W. Muller and D. M. Crothers, J. Mol. Biol., 28, 301 (1973).
16. T. D. Mokul'skaya, E. P. Smetanina, G. E. Myshko, M. A. Mokul'skaya, Mol. Biol., 9, 1514 (1975).
17. J. Marmur, J. Mol. Biol., 3, 208 (1961).