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Antitumor effects of binuclear ferrocene derivatives

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On the basis of an earlier model of chemical carcinogenesis, the antitumor activity of the mono-, bi- and poly-nuclear ferrocene derivatives ferricenium tri-iodide (1), ferricenium tetrachloroferrate (2), 1,1′-diethylferricenium tri-iodide (3), *N*-(ferrocenylmethyl)hexamethylenetetramine tetrafluoroborate (4), bis(ferrocenylmethyl)benzotriazolium tetrafluoroborate (5), bis(ferrocenyl-α-ethyl)benzotriazolium tetrafluoroborate (6) and bis(ferrocenylmethyl)-2-methylbenzimidazolium tetrafluoroborate (7), and the oligomer (—Fc—CH_2—$Fc^{+\cdot}$—CH_2—)$_{7-8}$-(PF_6)$_{7-8}$ (8) was studied *in vivo* (Fc = $C_{10}H_8Fe$). The tumor models studied included MCH-11 (mouse sarcoma induced by methylcholantrene), P-815 (mouse mastocytoma of DBA/2 origin) and virus-induced Raucher leukemia (RLV). The cytotoxic effects of these preparations were examined against *in vitro* cultured normal murine cells (line L-929). The binuclear ferrocene derivatives 5, 6 and 7 inhibited the development of experimental tumors in mice. Ferricenium tri-iodide (1) was effective in Rauscher leukemia. Kinetic dependencies for most complexes had a two-phase character: the region of inhibition of tumorogenesis was followed by a region in which the complexes accelerated the development of this process. The link between the structure of compounds 1–8 and their antitumor effects is discussed.

Keywords: Bis(ferrocenylalkyl)azolium cations, experimental tumors, virus-induced Rauscher leukemia, cytotoxicity, antitumor effect

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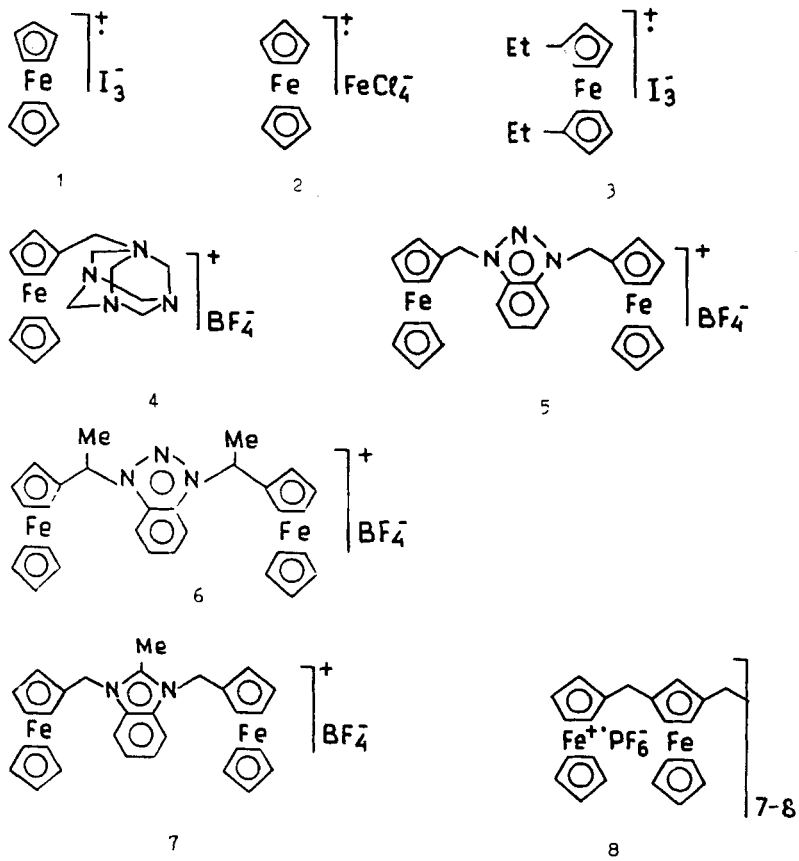
1 INTRODUCTION

In spite of the intensive development of the chemistry of metallorganic compounds during the last 40 years, work devoted to the use of these compounds in the chemotherapy of tumors has only recently begun to appear. In 1979 we made an attempt to analyze the molecular mechanism of tumor cell transformation induced by chemical carcinogens belonging to the classes of polycyclic aromatic hydrocarbons and azoaromatic derivatives.[1] Within the framework of the model of chemical carcinogenesis proposed in this work, the appearance of DNA sections free from nucleosome histones and containing gaps was the main result in the chain of primary damage to chromatin. It was supposed that the appearance of multiple fissures and gaps created an increased number of exchange processes in the genome, and initiated inappropriate recombination, repair, integration with the oncovirus genome and the activation of oncogenes. In the long run, it must lead to the transformation of cells into tumors.

Proceeding from these conclusions we formulated principles for the molecular design of antitumor reagents (or compounds). The capability of interfering with inappropriate intermolecular contacts between DNA fragments which contained single strand gaps was the main structural feature of the compounds. We decided to investigate the binuclear derivatives of ferrocene (containing functional groups) as one possible direction for research.[1] At the same time, other results were published, on the antitumor effects of alkylating agents of the sarcolysine type, modified by



Scheme 1

the introduction of a ferrocene substituent into the molecule.[2] The authors showed with different models of reinoculated tumors that these compounds did not display antitumor effects, either by themselves or under conditions of combined therapy.

In 1984 the antitumor activity of ferricenium salts with different anions was demonstrated.[3,4] However, using the model of Ehrlich ascites carcinoma (EAC), neither ferrocene nor inorganic iron(III) salts possessed the capability to inhibit tumor growth. Prior to these results, the number of metallocenes revealing cytostatic properties was increased using dihalides of the Cp_2MX_2 type (where M = Ti, V and X = halogen or any bidentate ligand).[5,6] These results prompted us to study the antitumor activity of our ferrocene compounds. In the present work we attempted to study the antitumor effects of mono- and polynuclear ferrocene derivatives, in order to answer the question of whether it was necessary to have a ferricenium form of the metallocene for antitumor properties. Also we decided to study the influence of ferrocene compounds upon reinoculated tumors of virus etiology.[7]

2 EXPERIMENTAL

2.1 Metallorganic compounds

In the present work three types of ferrocene derivatives (Scheme 1) were studied:

Ferricenium salts $[(C_5H_5)_2Fe]^+ I_3^-$ (**1**), obtained after recrystallization from acetone.[8]
Analysis: Found: Fe, 9.77; I, 67.11. Calcd for $C_{10}H_{10}FeI_3$: Fe, 9.85; I, 67.17%.

$[(C_5H_5)_2Fe]^+ FeCl_4^-$ (**2**), obtained from ferrocene according to standard method[19] with recrystallization under acid conditions.

1,1′-Diethylferricenium triiodide $[(C_5H_4C_2H_5)_2Fe]^+ I_3^-$ (**3**) was synthesized in the same way as the tri-iodide (**1**).
Analysis: Found C, 27.03; H, 2.83; Fe, 9.05; I,

60.96. Calcd for $C_{14}H_{18}FeI_3$ (**3**): C, 26.99; H, 2.91; Fe, 8.99; I, 61.12%.

Ferrocenylaklylated heterocycles (azoles) have been described previously[10] and were prepared by a published method.[11] In the present work we synthesized the mononuclear urotropine derivative—$(C_5H_5FeC_5H_4CH_2N_4C_6H_{12})^+BF_4^-$ (**4**) and binuclear salts of benzotriazole (**5**, **6**) and 2-methylbenzimidazole (**7**). Compounds **4**–**7** were obtained by ferrocenylalkylation with ferrocenylcarbinols of the respective heterocycles in the two-phase system chloromethylene/water:[10, 11]

N-(Ferrocenylmethyl)hexamethylenetetramine tetrafluoroborate (**4**).

Analysis: Found: C, 47.89; H, 5.79; N, 12.74; Fe, 13.21; B, 2.56. Calcd for $C_{17}H_{23}N_4FeBF_4$ (**4**): C, 47.92; H, 5.44; N, 13.15; Fe, 13.11; B, 2.54%.
IR (cm^{-1}): 3480, 3425, 3105, 2955, 1465, 1267, 1231, 1115, 1006, 923, 886, 817.
^1H NMR (acetone-d_6, δ): 4.19 (s, 2H); 4.25 (s, 5H); 4.39 (t, 2H); 4.56 (t, 3H); 4.62 ($s_{br.}$, 2H); 4.70 (s_{br}, 2H); 4.77 (s_{br}, 1H); 5.17 (s, 6H).

1*N*, 3*N*-Bis(ferrocenylmethyl)benzotriazolium tetrafluoroborate (**5**).
Analysis: Found: C, 54.80; H, 3.88; Fe, 19.04. Calcd for $C_{28}H_{26}Fe_2N_3BF_4$ (5): C, 55.77; H, 4.35; Fe, 18.52%. IR (cm^{-1}): 3100, 1656, 1614, 1452, 1108, 1006, 819.
^1H NMR (acetone-d_6): 4.27 (s, 14H); 4.67 (t, 4H); 6.14 (s, 4H); 8.00 (q, 2H); 8.35 (q, 2H).

The X-ray structure of the salt **5** was determined by M. Djafarov, A. S. Batsanov and Yu. T. Struchkov (in press).[17]

1*N*,3*N*-Bis(ferrocenyl-α-ethyl)benzotriazolium tetrafluoroborate (**6**).
Analysis: Found: C, 56.38; H, 4.57; N, 7.27; Fe, 17.36. Calcd for $C_{30}H_{30}Fe_2N_3BF_4$ (**6**): C, 57.10; H, 4.79; N, 6.66; Fe, 17.70%. IR (cm^{-1}): 3118, 3002. 2956, 1608, 1470, 1398, 1310, 1250, 1118, 1070, 1017, 830.

1*N*,3*N*-Bis(ferrocenylmethyl)-2-methylbenzimidazolium tetrafluoroborate (**7**).
Analysis: Found: C, 58, 53; H, 4.69; N, 4.31; Fe, 18.02. Calcd for $C_{30}H_{29}N_2Fe_2BF_4$ (**7**): C, 58.49; H, 4.74; N, 4.55; Fe, 18.13% IR (cm^{-1}): 3020, 1622, 1521, 1462, 1415, 1100, 1070, 1002, 822. ^1H NMR (acetone-d_6, δ): 3.20 (s, 3H); 4.20–4.55 (m, 18H); 5.65 (s, 4H), 7.58–8.14 (m, 4H).

The third type of compound studied comprised ferrocenylenemethylene oligomer (**8**)

$$[—(C_5H_4FeC_5H_4CH_2C_5H_4\overset{+\cdot}{F}eC_5H_4CH_2)—PF_6^-]_{7\text{-}8}$$

The initial compound $—[(C_5H_4FeC_5H_4CH_2)]—_{15}$ (**9**) has been obtained in argon by treatment of a chloromethylene solution of ferrocenylcarbinol ($FcCH_2OH$) with an aqueous solution of HBF_4 (48%). After chromatography (Al_2O_3, eluent benzene) a yellow powder was obtained, with quantitative yield.
Analysis: Found: C, 66.07; H, 5.52; Fe, 27.92. Calcd for $C_{11}H_{10}Fe$ (**9**): C, 66.37; H, 5.57; Fe, 28.06%.
Molecular weight: 2890.

This oligomer was then oxidized by $Fc^+ \cdot PF_6^-$ [12] to give

$$[—(C_{10}H_8FeCH_2C_{10}H_8\overset{+}{F}eCH_2)—PF_6^-]_{7\text{-}8}\ (\mathbf{8}).$$

Analysis: Found: C, 47.35; H, 3.74; Fe, 20.34; F, 20.65. Calcd for $C_{22}H_{20}Fe_2PF_6$ (**8**): C, 48.84; H, 3.73; Fe, 20.64; F, 21.07%.

2.2 Antitumor effects of compounds in tumor-bearing mice

2.2.1 Tumors induced by methylcholanthrene (tumor cell line MCH-11)

Sarcoma cells MCH-11, induced by methylcholanthrene, passed via C57Bl/6 mice, were inoculated intraperitoneally into female or male 8–10-week-old mice each week. To prepare MCH-11 tumor cells ascites were collected from the mice. Cells were separated by centrifugation at 150 *g* for 5 min at 4 °C. They were washed three times with Eagle's medium by centrifugation at 150 *g* for 5 min at 4 °C. Then they were suspended in the Eagle's medium so that the resulting suspension contained 5×10^5 cells cm^{-3}. Female 8–10-week-old mice were injected subcutaneously or intraperitoneally with 0.2 cm^3 (containing 10^5 cells) of this suspension. Hence the development of solid or ascites tumors took place. All tumor-bearing mice were injected intramuscularly with compounds **1**–**8** after 24 h at doses of 10 or 100 μg/mouse. The control and reference groups contained 18–20 animals. The antitumor effects of the compounds were estimated according to the time of the tumor development and the calculation of lifespan.

2.2.2 Mastocytoma P-815 of DBA/2 origin

P-815 cells were maintained in suspension in an RPMI-1640 medium supplemented with 10%

fetal calf serum, penicillin (50 units cm^{-3}) and streptomycin (50 μg cm^{-3}) at 37 °C. The cells were washed by centrifugation at 150 *g* for 5 min at 4 °C and resuspended in the RPMI-1640 medium. Then 2×10^5 cells (0.2 cm^3) were injected subcutaneously into the back of 8–10-week-old female DBA/2 mice. Compounds **5**, **7**, **8** (10 μg/mouse) and compound **1** (100 μg/mouse) were administered intramuscularly after 48 h.

2.2.3 Rauscher viral leukemia

An inoculum (0.1 cm^3) of a 10-fold dilution of a leukemic plasma was injected intraperitoneally into 8–10-week-old BALB/C inbred mice, either male or female (5–7 animals per batch). Compounds **1–8** (10 and 100 μg/mouse) were administered intramuscularly 48 h after this injection. Compound **1** was administered intramuscularly at doses of 10, 100 and 500 μg/mouse 24 and 48 h after single or triple virus inoculation at intervals of 4–5 days.

At 21 days post-infection, the mice were sacrificed and their spleens were weighed individually on a torsion balance. The degree of splenomegaly is dependent on the dose of virus and may be used to measure the amount of virus in infected mice. Mice with spleens exceeding 300 mg were considered leukemic.[13] The virus titer was calculated according to Reed and Muench.[18]

In vivo experiments (2.2.1–2.2.3) complexes **1**, **5**, **6**, **7** and **8** were dissolved in DMSO (20 mg cm^{-3}), complex **3** in ethanol (20 mg cm^{-3}) and diluted with distilled water to give a final range of concentrations 100–1000 mkg cm^{-3}. Complexes **2** and **4** were dissolved in distilled water.

2.2.4 *In vitro* test

For the assessment of cytotoxicity, mouse fibroblasts (line L-929) were used. L-929 cells were cultured in 96-well microplates ('Nunc') in a 199 medium supplemented with 10% fetal calf serum, penicillin (50 units cm^{-3}), and streptomycin (50 μg cm^{-3}) at 37 °C. Measurement of cytotoxicity was based on alteration of normal cell morphology. To evaluate this parameter, parallel cell cultures were treated with various concentrations (5–500 μg cm^{-3}) of complexes **1–8**. These cultures were examined microscopically after 24 and 48 h. Disruption of cell monolayers, e.g. rounding up, darkness or detachment of cells, was considered as evidence of cytotoxicity. Complexes **1**, **5**, **6**, **7** and **8** were dissolved in DMSO (20 mg cm^{-3}). Complexes **2** and **4** were dissolved in distilled water, complex **3** in ethanol. The solutions were diluted with 199 medium supplemented with 2% fetal calf serum to give a final range of concentrations 5–500 mkg cm^{-3}.

RESULTS AND DISCUSSION

3.1 Cytotoxicity of compounds 1–8

These results are summarized in Table 1.

It is clear that in a concentration of 500 μg cm^{-3}, all substances except ferricenium ferrichloride (**2**) are toxic. This latter fact is of note since the molar concentration of this compound is 1.3–1.6 times higher than those of other compounds. At 50 μg cm^{-3} concentration all preparations are nontoxic except the binuclear derivative of 2-methylbenzimidazole (**7**). It is not easy to compare these concentration ranges with those studied *in vivo*. However, assuming that the mouse mean volume is 20 cm^3 and neglecting dry weight, it is possible to estimate that a dose of 10–100 μg/mouse is equivalent to the 0.5–5.0 μg cm^{-3} concentrations used in the cell culture experiments. Thus, the experiments on

Table 1 Cytotoxic effects of compounds **1–8** in L-929 cells

Concentration of the ferrocene derivatives (μg cm^{-3})	Ferrocene derivatives: **1**	**2**	**3**	**4**	**5**	**7**	**8**
5	nr[a]	nr	nr	nr	nr	nt[b]	nr
50	nt	nt	nt	nt	nt	t[c]	nt
	8.81[d]	13.00	8.00	9.67	9.36	8.99	9.24
500	t	nt	t	t	t	nr	t

[a] nr, not researched at 5 μg cm^{-3}. [b] nt, non toxic. [c] toxic. [d] Molar concentration of ferrocene derivatives $\times 10^{-11}$.

animals were carried out with concentrations which are 10–100 times less than the toxic ones.

3.2 Influence of compounds on the development of tumors induced by MCH-11 cells

Kinetic dependence curves of mice mortality versus time [function $N=f(t)$ where N is the number of deceased animals and t is time] are given in Figs 1–3. Similar curves for the number of surviving animals [$M=f(t)$ where M is the number of surviving mice at a given time] are shown in Figs. 4–6. Curves for the proportions of survivors in a group [$M/M_0=f(t)$ where M_0 is the number of surviving mice in a group] are presented in Figs 7–9. Semi-logarithmic plots corresponding to the last dependences, i.e. $\ln M/M_0 = f(t)$, are shown in Figs 10–12.

It can be seen that compounds **3** and **4** in practice do not prolong lifespan (see Figs 1–3). Moreover, it is possible that there is a weak tendency toward accelerating tumor growth. However, the compounds **5**, **7** and **8** significantly prolong the lifespan of experimental animals with developed solid tumors.

For Figs 10–12, it should be borne in mind that analysis is difficult due to population heterogeneity of the experimental groups. It is clear that while survival characteristics of the second and third control groups (Figs 11 and 12) have linear character, the first control group (Fig. 10) is characterized by three linear regions. This fact should be taken into account on analyzing the rate constants for animals mortality, which are given in Table 2.

Comparison of rate constants on the first part of Fig. 10 (Table 2) shows that, in the first group, compounds **4**, **6** and especially **5** seem to be most effective. Using the latter compound, e.g. the binuclear ferrocenylalkylated benzotriazole salt, more than 30-fold inhibition of tumor growth at a dose of 10 μg/mouse is achieved. Table 2 also shows that a high degree of inhibition is reached when compound **8** is used. It should be noted that the most effective compounds in relation to tumor MCH-11 inhibition, viz. compounds **5**, **6** and **8** are di- or multi-nuclear derivatives of ferrocene. Only **4**, a ferrocenylalkylated derivative of urotropin, is an exception.

On the contrary, ferricenium salts accelerated the development of a solid MCH-11 tumor.

The unexpected connection between the dose used of a compound and its effect, in the case of compounds **4**, **5** and **8** should be noted. Increase in the dose of these compounds from 10 to 100 μg/mouse leads to a decrease of inhibition (Table 2). As far as compound **6** is concerned, the dose–effect dependence has a usual character.

In Table 3 data relating to the commencement of the animal mortality—the day and number of deaths—are presented. It is seen that the substances studied very noticeably decrease the number of animal deaths, but they have very little effect on the induction period. Moreover, the same substances seem to be most effective, i.e. compounds **4**, **5**, **6** and **8**. Compound **7** (a binuclear derivative of 2-methylbenzimidazole) may also be included in this list. It should be taken into account that on this parameter the inversion of the normal run of effect–dose dependence for compounds **4** and **6** is observed.

As noted above, kinetic dependences have a two-phase character for most substances. The inhibition region of tumor development is substituted by a region in which the preparation accelerates its development (see Figs 10–12). Therefore, it was of interest to compare transition from one regime to another. As seen from the data on transition lines in Table 4, the compounds of the ferrocene group significantly delay the time of tumor development. Compounds **1–3** turn out to be most effective, e.g. ferricenium salts but not binuclear ferrocenylalkylated azolium cation salts, as happened in the previous case.

3.3 Influence of the ferrocene complexes upon the development of P-815 mastocytoma

On injection of compounds **1**, **5**, **7** and **8** it was established that the time of appearance of tumors and their rate of development did not differ from control groups. Thus, ferricenium salts as well as diferrocenylalkylated azole cations turned out to be ineffective in relation to mastocytoma P-815.

3.4 Influence of compounds 1–8 on Rauscher leukemia virus (RLV)

All the compounds of this series, excluding ferricenium tri-iodide (**1**), did not affect the synthesis of RLV. Compound **1** in a dose of 100 μg/mouse reduced the virus titer from $10^{-4.3}ID_{50}$ in the control group to $10^{-3.6}ID_{50}$. The increase of compound dose to 500 μg/mouse did not result in additional reduction of the virus titer. Triple injections of compound **1** in doses of 100 μg/

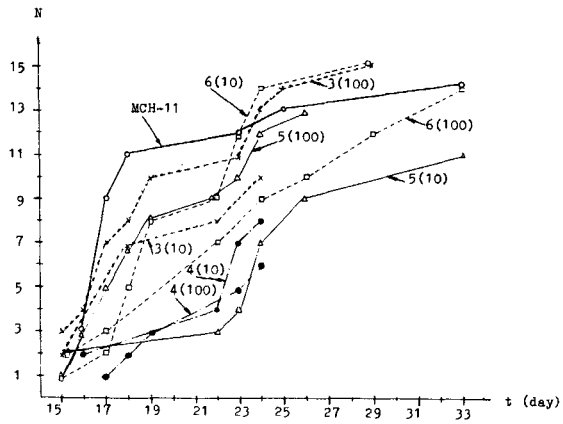


Figure 1 Dependence of the number of deceased animals (N) upon the time (t) from inducing the MCH-11 cells. The numbers on the curves indicate the compound (**3**, **4**, **5** and **6**) and the dosage (10 or 100 μg/mouse). Curve MCH-11 control group 1.

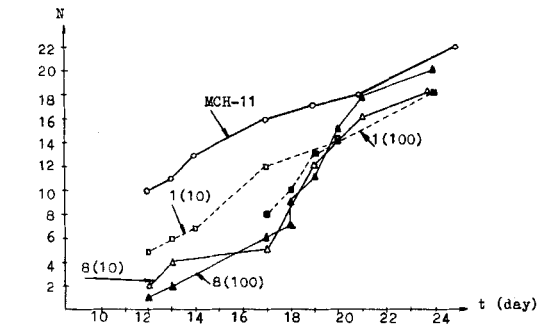


Figure 3 Dependence of the number of deceased animals (N) upon the time (t) from inducing the MCH-11 cells. Curve MCH-11, control group 3. The other curves are labelled as described in Fig. 1, for compounds **1** and **8**.

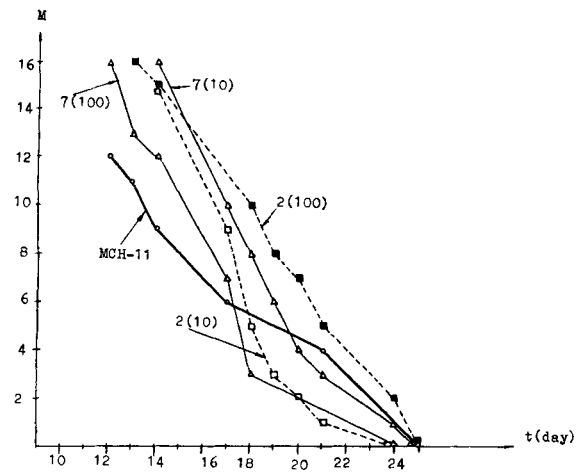


Figure 5 Dependence of the number of surviving animals (M) upon the time (t) from inducing the MCH-11 cells.

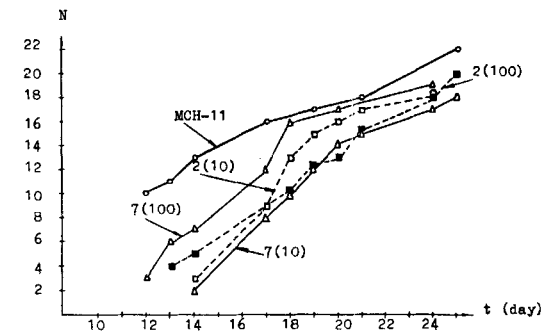


Figure 2 Dependence of the number of deceased animals (N) upon the time (t) from inducing the MCH-11 cells. Curve MCH-11, control group 2. The other curves are labelled as described in Fig. 1, for compounds **2** and **7**.

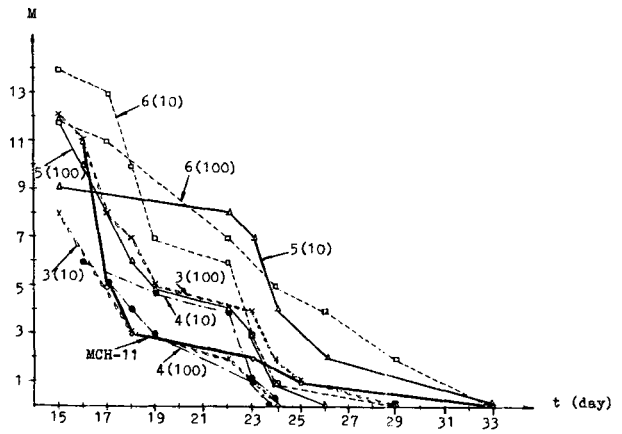


Figure 4 Dependence of the number of surviving animals (M) upon the time (t) from inducing the MCH-11 cells.

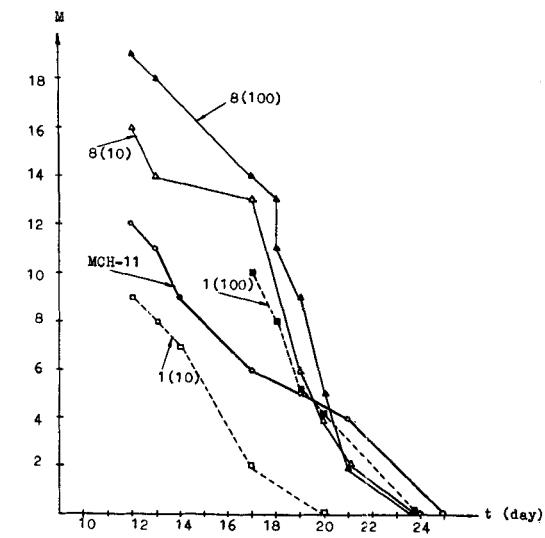


Figure 6 Dependence of the number of surviving animals (M) upon the time (t) from inducing the MCH-11 cells.

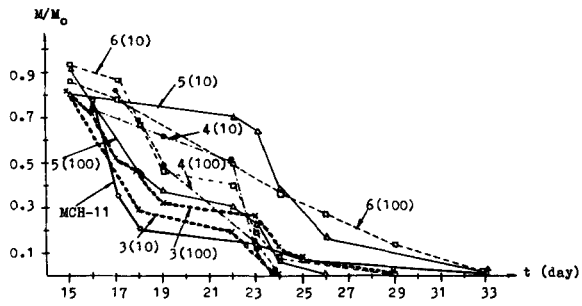


Figure 7 Dependence of the proportion of surviving animals (M/M_0, where M is the number of surviving animals, M_0 the number of mice in a group) upon the time (t) from inducing the MCH-11 cells.

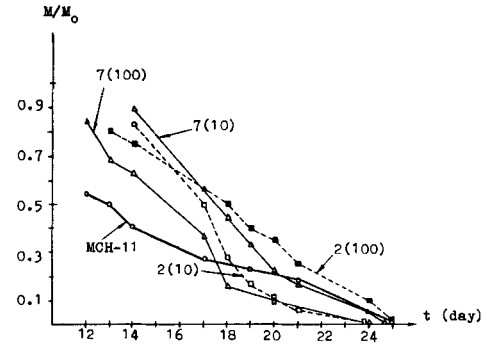


Figure 8 Dependence of the proportion of surviving animals (M/M_0) upon the time (t) from inducing the MCH-11 cells.

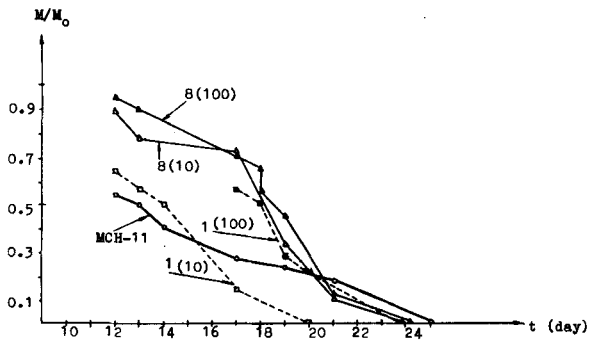


Figure 9 The dependence of the proportion of surviving animals (M/M_0) upon the time (t) from inducing the MCH-11 cells.

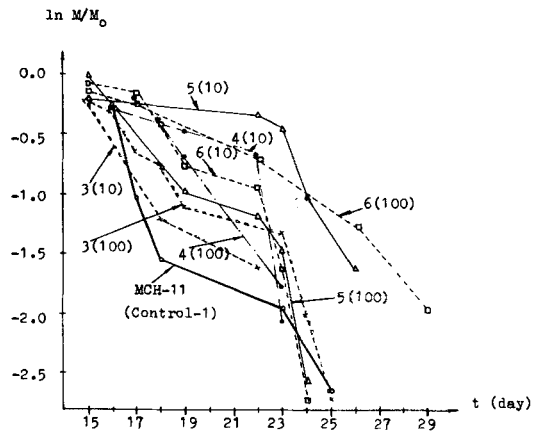


Figure 10 Semi-logarithmic plot of the dependence of the proportion of surviving animals upon the time (t) from inducing the MCH-11 cells.

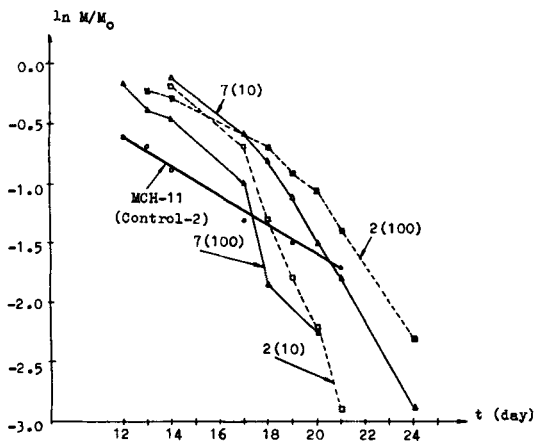


Figure 11 Semi-logarithmic plot of the dependence of the proportion of surviving animals upon the time (t) from inducing the MCH-11 cells.

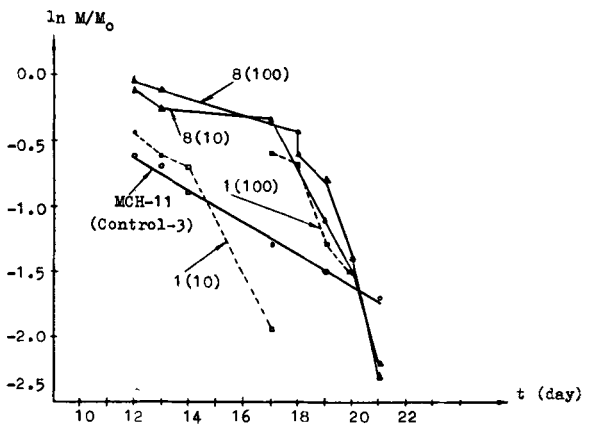


Figure 12 Semi-logarithmic plot of the dependence of the proportion of surviving animals upon the time (t) from inducing the MCH-11 cells.

Table 2 The rate constants[a] of survival of mice bearing the MCH-11 tumor

No.	Ferrocene compound	Dose (μg/mouse)	k_1	k_2	k_3	$k_{1(control)}/k_1$
1	Control 1		0.65	0.08	0.34	1
2	**3**	10	0.32	0.10	—	2.03
3	**3**	100	0.22	0.05	0.77	2.95
4	**4**	10	0.06	1.40	—	10.83
5	**4**	100	0.16	0.27	—	4.06
6	**5**	10	0.02	0.20	—	32.50
7	**5**	100	0.23	0.07	1.08	2.83
8	**6**	10	0.32	0.06	1.09	2.03
9	**6**	100	0.09	0.23	—	7.22
10	Control 2		0.12	—	—	1
11	**2**	10	0.17	0.68	—	0.70
12	**2**	100	0.10	0.30	—	1.20
13	**7**	10	0.07	0.42	—	1.71
14	**7**	100	0.16	0.60	—	0.75
15	Control 3		0.15	—	—	1
16	**1**	10	0.13	0.41	—	1.15
17	**1**	100	0.11	0.17	—	1.36
18	**8**	10	0.03	0.40	—	5.00
19	**8**	100	0.06	0.60	—	2.50

[a] For definition of rate constant here, the tangent of each curve in Figs. 10–12 was determined $\left(\tan \frac{\ln M/M_0}{t}\right)$

Table 3 Details of mortality of mice bearing MCH-11 tumors

Ferrocene complex	Dose (μg/mouse)	Day of commencement of mortality of first mouse	No. of mice deceased on first day, A	No. of mice in the group, *B*	Percentage of *A* relative to *B*
Control 1		16	3	14	21.43
3	10	15	2	10	20.00
3	100	15	3	15	20.00
4	10	16	1	8	12.50
4	100	17	1	6	16.67
5	10	15	2	11	18.18
5	100	15	1	13	7.69
6	10	15	1	15	6.67
6	100	15	2	14	14.29
Control 2		12	10	22	45.45
2	10	14	3	18	16.67
2	100	13	4	20	20.00
7	100	12	3	19	15.79
7	10	14	2	18	11.11
Control 3		12	10	22	45.45
1	10	12	5	14	35.71
1	100	17	8	18	44.44
8	10	12	2	18	11.11
8	100	12	1	20	5.00

Table 4 Time change of kinetic regimes

No.	Ferrocene compound	Dose (μg/mouse)	T-1[a]	T-2[b]
1	Control 1		16	23
2	**3**	10	18	—
3	**3**	100	19	23
4	**4**	10	—	22
5	**4**	100	17	—
6	**5**	10	—	23
7	**5**	100	19	23
8	**6**	10	19	22
9	Control 2		12	—
10	**2**	10	17	—
11	**2**	100	19	—
12	**7**	10	17	—
13	**7**	100	17	—
14	Control 3		12	—
15	**1**	10	14	—
16	**1**	100	18	—
17	**8**	10	17	—
18	**8**	100	18	—

[a] T − 1 designates the day on which the change of the kinetic regime of tumor development occurs. [b] T − 2 is the day on which the transition of the animal group to the following kinetic regime takes place.

mouse at intervals of 4–5 days also decreased the virus titer to $10^{-3.66}ID_{50}$ in comparison to $10^{-4.5}ID_{50}$ in the control group. Earlier injection of compound **1** (in 24 and not in 48 h) did not lead to reduction of the virus titer ($10^{-4.83}ID_{50}$ against $10^{-4.37}ID_{50}$ in the control group).

Thus, in relation to developing Rauscher leukemia, the binuclear ferrocenylalkyl-substituted derivatives of azoles turned out to be ineffective. On the other hand, ferricenium salts—ferricenium ferrichloride (**2**) and 1,1′-diethylferricenium tri-iodide (**3**)—were also ineffective. It may be supposed that in this case only the combination of the unsubstituted ferricenium cation and the I_3 anion gives an effect, as occurs in compound **1**.

Similar results were obtained on studying the lifespan of animals. The injection of ferricenium tri-iodide gives some deceleration of leukosis development.

3.5 Structure of ferrocene compounds 1–8 and their antitumor effect

Thus, a certain effectiveness of complexes 4–8 in relation to solid MCH-11 tumor was discovered in the compounds examined. In relation to Rauscher leukosis only compound **1** displayed such a property.

As it was already noted above, compounds **5–7** are azolium cations in which a heterocyclic bridge links two ferrocenylalkyl nuclei. In these cases the tetrafluoroborate anion plays a counterion role. Compound **4** is structurally similar to these substances. This is a salt, the cation of which is actually a ferrocenylalkylated derivative of urotropin. It differs from compounds of the previous group (**5–7**), since its cation has only one ferrocenylaklyl nucleus.

Thus, compounds **4–7** are not salts of ferricenium as are compounds **1–3** and **8**. Compound **8** as mentioned before, is an oligomer in which ferrocene and ferricenium nuclei alternate.

At the present time it would be premature to make any definite conclusion in relation to the molecular mechanism responsible for the activity of compounds **4–8.** However, some preliminary conclusions can be made.

First, it should be noted that in accordance with previous suppositions[1] binuclear ferrocene derivatives with functional groups do actually display antitumor activity. Moreover, this activity is higher than that of the mononuclear derivatives. Thus, the results obtained in the present work confirm the idea that binuclear ferrocene derivatives with different substituents which are capable of coordinating with reactive centers of DNA nucleotides, can hamper recombination process of tumor character. It has to be asked if there is a reason to suppose that the compounds under study actually reach the genome. Also, if they reach the nucleus of tumor cells, do they still have their original molecular structure?

We can, with some degree of certainty, give a positive answer to this question. The two-phase kinetic dependence of effect upon time (Figs. 10–12) is worth mentioning. As noted above, the ferrocene complexes initially play the role of tumor inhibitors and at later stages, on the contrary, they accelerate tumorigenesis. We think that a natural explanation of this effect would be a supposition that at the first stage the complexes in their initial form inhibit tumor development. At the same time their gradual metabolic transformation takes place, which is caused by the oxidation of ferrocene nuclei and substitution of cyclopentadienyl ligands. In addition, released iron ions are transported into the cytoplasm, and may activate redox processes in cells. These circumstances would lead to the acceleration of tumor development at the second stage, since it is

well known that a tumor is a trap not only for glucose (for example) but also for iron.[14]

Another question also arises: where do the compounds interact with the DNA in the target cells?

Three types of possible interactions can be named. First, electrostatic interaction of ferrocene- or ferricenium-containing cations with phosphorus anions of detached ends of DNA, having an excess of negative charge; or secondly, intercalation (or to be more exact, partial intercalation) of cyclopentadienyl ligands of ferrocene nuclei between nucleic bases. [It is worth mentioning that the interligand distances in ferrocene derivatives (3.5 Å; 0.35 nm) coincide with distances between planes of nucleic bases of DNA.] Finally, the third possible type of interaction can be by contacts between nucleophile centers of nucleic bases and the central atoms of the ferrocene nuclei. Similar interactions are also known in the literature.[15]

Generally speaking, the data obtained testify to the fact that the degree of oxidation of the central atom does not play a principal role in displaying antitumor properties. Only the salt nature of the compounds seems to be important. From the viewpoint of transport properties of molecules, the lipophilic properties of the ferrocene nucleus are preserved after its transformation into the ferricenium form.[16] Therefore, it may be expected that all of compounds **1–8** are capable of penetrating membrane structures. On the other hand, their salt nature also allows the possibility of migrating in polar media. However, we consider it as a natural supposition that the primary metabolism of ferrocene complexes must lead to their reaching the cell nuclei in the oxidized ferricenium form. This is most probable since the compounds are injected intramuscularly. One more question arises in connection with the ferricenium tri-iodide effect upon Rauscher leukosis and its absence on using binuclear derivatives. One possible explanation of this phenomenon may be attributed to the increased immunogenicity of the larger molecules of the azolium salts (**5–7**). The activation of the animals' immune system by freshly reinoculated Rauscher leukosis cells can lead to a great part of the preparation being metabolized by macrophages on the approaches to target cells. It is possible that the anomalies in dose–effect dependences, which we discovered in some of these compounds, were connected with this effect. A large dose causes enhanced immune response and, as a consequence, earlier destruction of the compounds.

4 CONCLUSIONS

Thus, the conclusion can be made that binuclear ferrocene derivatives actually inhibit the development of experimentally reinoculated tumors. Moreover, ferricenium salts turn out to be less effective than derivatives having neutral ferrocene nuclei. We plan to broaden the number of polynuclear ferrocene derivatives and to study their effect in combinations in future work.

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REFERENCES

1. Babin, V N, Dubinin, A V, Raevskii, P M, Sviridov, A F, and Sherman, A L Model of chemically-induced carcinogenesis. In: *Modeli. Algoritmy*, Prinyatie Reshenii, Nauka, Moscow, 1979, pp 153–167
2. Yashchenko, G N, Shasmurina, A A, Anoshina, G M, Gorelova, L A, Evstigneeva, N G, Alekseeva, L V and Radina, L B *Khim.-Farm.* Zh., 1978, 12: 68
3. Köpf-Maier, P, Köpf, H and Neuse, E W *Angew. Chem.*, 1984, 96: 446
4. Köpf-Maier, P, Köpf, H and Neuse, E W *J. Cancer Res. Clin. Oncol.*, 1984, 108: 336
5. Köpf, H and Köpf-Maier, P *Angew. Chem.*, 1979, 91: 509
6. Köpf-Maier, P, Wagner, W, Hesse, B and Köpf, H *Eur. J. Cancer*, 1981, 17: 665
7. Snegireva, A E, Shaposhnikova, G M, Ignatenko, M A, Shevlyaghin, V Ya, Dobrunin, Ya V, Nikolaeva, T T, Berovskii, Yu V and Karabach, O S *Bull. Experiment. Biol. Med.*, 1990, 102: 178
8. Nesmeyanov, A N, Yurjeva, L P, Materikova, R B and Hetnarski, B *Izv. Akad. Nauk SSSR, Ser. Khim.*, 1965, 4: 731
9. Nesmeyanov, A N, Perevalova, E G and Yurjeva, L P *Chem. Ber.*, 1960, 93: 2729
10. Kochetkova, N S, Boev, V I, Popova, L V and Babin, V N *Izv. Akad. Nauk SSSR, Ser. Khim.*, 1985, 6: 1397
11. Kochetkova, N S, Boev, V I, Babin, V N, Materikova, R B, Popova, L V and Bondarenko, V M USSR Patent SU 1 320 212, 25 Sept 1985
12. Neuse, E W and Khan, F B D *Macromolecules*, 1986, 19: 269
13. Chirigos, M A *Cancer Res.*, 1964, 24. 1035
14. Volleman, M *Biochemistry of Brain Tumors*, Mir, Moscow, 1977
15. Babin, V N, Belousov, Yu A, Gumenyuk, V V, Salimov, R M, Materikova, R B and Kochetkova, N S *J. Organomet. Chem.*, 1983, 241: C41
16. Shinbo, T, Sugiura, M, Kamo, N and Kobatake, Y *J. Membrane Sci.*, 1981, 9: 1
17. Popova, L V, Boev, V I, Babin, V N, Nekrasov, Yu S, Djafarov, M, Batsanov, A S and Struchkov, Yu T *Organomet. Chem. in the USSR*, in press
18. Reed, L Y and Muench, H H *J. Am. Hyg.*, 1938, 27: 493
19. Neuse, E W *Transition Met. Chem.*, 1985, 10: 135