Tumor Inhibition by Ferricenium Complexes: Systemic Effect *in vivo* and Cell Growth Inhibition *in vitro*

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The antiproliferative activity of divers ferricenium complexes $[Cp_2Fe]^+X^-$ ($X^- = [CCl_3COO]^- \cdot CCl_3COOH$ (**I**); $X^- = [CCl_3COO]^- \cdot 2\ CCl_3COOH$ (**II**); $X^- = \frac{1}{2}\ [Cl_3FeOFeCl_3]^{2-}$ (**III**); $X^- = [FeCl_4]^-$ (**IV**); $X^- = [2,4,6\text{-}(NO_2)_3C_6H_2O]^-$ (**V**)) was investigated against solid, subcutaneously growing Ehrlich ascites tumor (EAT) *in vivo* as well as against EAT cells cultivated *in vitro* as permanent suspension culture. *In vivo*, triple intraperitoneal injections of the complexes **II** (3×200 mg/kg), **III** (3×100, 140, 180 mg/kg) or **IV** (3×160 mg/kg) markedly suppressed tumor development thus that the sizes of treated tumors were reduced to 42–48% related to control tumors (100%); these results point to the systemic character of the antitumor action by ferricenium complexes *in vivo*. *In vitro*, all ferricenium complexes inhibited cellular proliferation to an equal extent; application of 10^{-5} M diminished the increase in cell number by 20–40%, application of 10^{-4} M resulted in a total cessation of cellular proliferation. In comparison to *cis*-diamminedichloroplatinum(II), the cell growth-inhibiting effect of ferricenium complexes was less pronounced and required 10- to 50-fold higher concentration levels to evoke equivalent cytostasis.

Introduction

By the discovery of antitumor efficacy for *cis*-diamminedichloroplatinum(II) [1], intense studies searching for other inorganic and organometallic antitumor agents have been stimulated. These investigations revealed a series of unexpected results: It was shown that not only numerous complexes containing platinum or other platinum metals exhibit antitumor properties, but that also other quite differing compounds are characterized by antiproliferative activity: (i) main group compounds such as inorganic gallium salts [2] or organometallic tin compounds [3]; (ii) early transition metal complexes containing titanium or vanadium [4]; and (iii) "middle" transition metal compounds represented by some ferricenium complexes [5].

The antitumor activity of the latter iron-containing compounds was recently detected against fluid Ehrlich ascites tumor (EAT) in mice [5]; this activity is surprising and remarkable because the ferricenium complexes fundamentally differ from known non-organic antitumor agents by their ionic character and by the absence of a *cis*-dihalometal moiety within the molecules.

Abbreviations: Cp, C_5H_5, cyclopentadienyl ring ligand; EAT, Ehrlich ascites tumor.

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In the present investigation, we continued the studies into the cytostatic properties of ferricenium complexes and examined (i) if the antitumor properties of these compounds are based on a systemic effect and (ii) if the antineoplastic activity shown *in vivo* is parallelled by cell growth-inhibiting effectiveness *in vitro*.

Materials and Methods

Substances

The ferricenium complexes*
$[Cp_2Fe]^+[CCl_3COO]^- \cdot CCl_3COOH$ (**I**; ferricenium trichloroacetate, mono(trichloroacetic acid) solvate; [6]), $[Cp_2Fe]^+[CCl_3COO]^- \cdot 2\ CCl_3COOH$ (**II**; ferricenium trichloroacetate, bis(trichloroacetic acid) solvate; [6]), $[Cp_2Fe]_2^+[Cl_3FeOFeCl_3]^{2-}$ (**III**; ferricenium μ-oxo-bis[trichloroferrate(III)]; [7]), $[Cp_2Fe]^+[FeCl_4]^-$ (**IV**; ferricenium tetrachloroferrate(III); [8]), $[Cp_2Fe]^+[2,4,6\text{-}(NO_2)_3C_6H_2O]^-$ (**V**; ferricenium picrate; [9]) were prepared according to the cited methods and purified as required.

In vivo testing

The investigations *in vivo* were performed against solid EAT growing subcutaneously in the nuchal region of mice.

* The substances were kindly provided by Professor Dr. E. W. Neuse, Johannesburg, Republic of South Africa.

For tumor transplantation, ascitic tumor was harvested from donor mice bearing fluid EAT for 8 days and diluted with saline (1:7, v:v). Volumes containing about 10^7 tumor cells were then inoculated subcutaneously into the nuchal region of a total of 173 mice (Table I). The day of tumor transplantation was defined as day 0 of the experiment. On days 1, 3, and 5, the different doses of ferricenium complexes (Table I) were diluted in saline (0.4–0.5 ml per animal) and administered intraperitoneally; each dose group consisted of 10 mice. The 33 animals of the control groups a, b, and c (Table I) only received injections of saline (0.4–0.5 ml) without drug addition on days 1, 3 and 5. Deaths occurring within 8 days after tumor transplantation were considered as toxic deaths due to substance toxicity.

On day 9, all animals were killed under ether anesthesia, the tumors were removed and weighed with an accuracy of ± 1 mg. The T/C ratios were then calculated by relating the mean tumor weights of the treated groups to that of the appertaining control group.

In vitro testing

The cell growth-inhibiting activity of ferricenium complexes was investigated against EAT cells growing *in vitro* as permanent suspension culture; details of cell culture techniques have been described before [10].

To these cell cultures containing about 4×10^4 cells/ml at the beginning of the experiment, the ferricenium complexes were added in final concentrations of 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} M. Because of the good water solubility of the substances, no addition of a solute, *e.g.*, of dimethylsulfoxide, was necessary. Four bottles were prepared for each concentration stage. Further four untreated cell populations served as controls for each ferricenium compound.

Beginning immediately after drug addition, every 24 h up to 96 h, 0.5 ml of the cell suspensions were removed from each bottle and the cell number per ml was counted by use of a hematocytometer. The increase in cell number per ml, expressed as a percentage of the cell number per ml, which was measured immediately after addition of the antitumor agents, was then appointed for each cell population at 24, 48, 72 and 96 h. After determining the mean values and standard deviations for each concentration stage, the T/C ratios were calculated by relating as percentage the increase in cell number of treated populations to that of the appertaining control population at the various times of exposure. The values determined at 72 h are given in Table II.

Results

The *in vivo* data of the present study show some of the ferricenium complexes investigated to be capable of inhibiting the growth of solid EAT (Table I): Treatment with the trichloroacetate (**II**; Fig. 1), μ-oxo-bis[trichloroferrate] (**III**) and tetrachloroferrate (**IV**) derivatives inhibited tumor development in dependence upon the doses applied. After treatment with middle doses of **II**, **III** or **IV**, the T/C ratios amounted to 48–62%, after treatment with high doses to 42–44%. This means that the tumor size was reduced by administration of **II**, **III** or **IV** to values smaller than the half of the appertaining control tumors. In this connection it is worth mentioning

Table I. *In vivo* testing of ferricenium complexes **I–V** against solid EAT.

Compound	Applied doses[a] [mg/kg]	Toxic deaths/ animals treated	Tumor weight[b] [g]	T/C [%]
I	3× 40	–/10	0.75 ± 0.32	104
	3× 80	1/10	0.78 ± 0.26	108
Control a	–	–/11	0.72 ± 0.44	–
II	3×100	–/10	0.24 ± 0.11	56
	3×150	–/10	0.22 ± 0.10	52
	3×200	1/10	0.18 ± 0.11	42
Control b	–	–/11	0.43 ± 0.25	–
III	3×100	–/10	0.36 ± 0.14	48
	3×140	–/10	0.36 ± 0.09	48
	3×180	2/10	0.33 ± 0.11	44
Control c	–	–/11	0.75 ± 0.50	–
IV	3× 80	–/10	0.33 ± 0.29	87
	3×120	–/10	0.23 ± 0.08	61
	3×160	1/10	0.19 ± 0.09	44
Control b	–	–/11	0.43 ± 0.25	–
V	3×100	–/10	0.79 ± 0.33	110
	3×150	–/10	0.69 ± 0.39	96
	3×200	2/10	0.60 ± 0.21	83
Control a	–	–/11	0.72 ± 0.44	–

[a] The highest doses corresponded to LD_{10} regimens.
[b] Given are mean values and standard deviations.

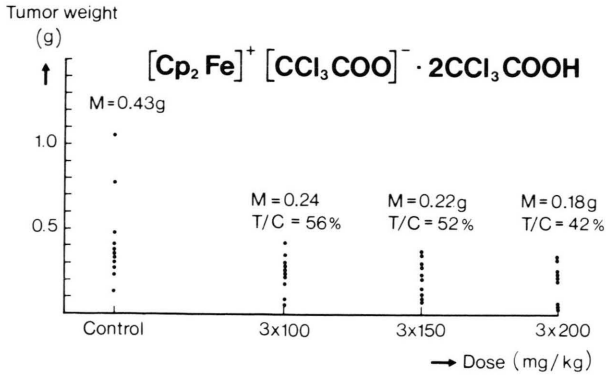

Fig. 1. Ranged tumor weights of solid EAT grown in the nuchal region after intraperitoneal treatment with ferricenium trichloroacetate (**II**) on days 1, 3 and 5 after tumor transplantation. The tumors were removed and weighed on day 9. M = mean value.

that a T/C ratio of 50% is generally considered to be the crucial level for showing activity against solid tumor systems [11].

On the other hand, the ferricenium complexes **I** and **V** failed to display growth-suppressing activity against solid EAT (Table I). Because application of the highest doses caused the death of 10–20% of treated animals due to substance toxicity, a further increase in doses was not feasible.

The *in vitro* data characterizing the cell growth behavior during exposure of EAT cells to the ferricenium complexes **I**–**V** and to *cis*-diammine-dichloroplatinum(II) are summarized in Table II; in Fig. 2, typical growth curves are shown under the influence of **I**.

All ferricenium complexes which were investigated caused very similar effects: Concentrations of 10^{-4} M drastically stopped any cellular proliferation, they obviously even destroyed cells which were present at the beginning of the experiment and gave rise to a continuous decrease in cell number resulting in T/C values smaller than 0% at 72 h. Reducing the applied concentrations of ferricenium complexes by

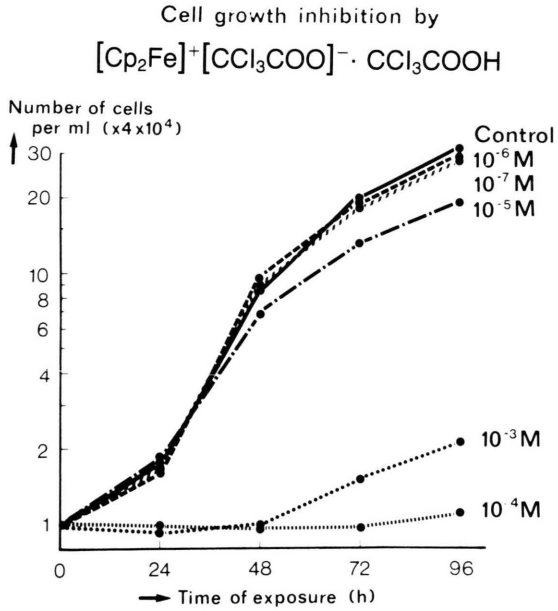

Fig. 2. Influence of a continuous, 96-h-lasting exposure of EAT cells to various concentrations (given on the right) of ferricenium trichloroacetate (**I**) on cellular proliferation. Beginning of exposure at 0 h. Values given are mean values obtained from four cell populations each.

Table II. *In vitro* testing of ferricenium complexes **I**–**V** against EAT cells.

Compound	Concentration (M) 0 (Control)	10^{-7}	10^{-6}	10^{-5}	10^{-4}
I	1880 ± 255	1760 ± 182 (94%)	1830 ± 80 (97%)	1210 ± 112 (64%)	− 25 ± 27 (< 0%)
II	1300 ± 315	1200 ± 162 (92%)	1100 ± 268 (85%)	930 ± 108 (72%)	− 12 ± 79 (< 0%)
III	1630 ± 215	1600 ± 137 (98%)	1800 ± 142 (110%)	1320 ± 256 (81%)	− 33 ± 74 (< 0%)
IV	1150 ± 108	860 ± 98 (75%)	960 ± 98 (83%)	710 ± 96 (62%)	− 8 ± 77 (< 0%)
V	1010 ± 127	680 ± 70 (67%)	700 ± 79 (69%)	760 ± 82 (75%)	5 ± 64 (< 1%)
cis-diammine-dichloro-platinum(II)	1340 ± 91	1360 ± 131 (101%)	300 ± 94 (22%)	0 ± 36 (0%)	− 60 ± 62 (< 0%)

The evaluated parameter is the increase in cell number (mean values and standard deviations) at 72 h after drug addition, expressed as a percentage of the cell number per ml at 0 h. The values in parentheses represent the appertaining T/C values at 72 h.

factor 10 to 10^{-5} M, cellular proliferation was yet diminished (Fig. 1); the cell numbers which were counted at 72 h amounted to 60–80% related to those of control populations (Table I). After administration of 10^{-6} M, finally, only in the case of **II**, **IV** and **V**, the cellular proliferation was slightly suppressed to 70–85%.

Under the influence of *cis*-diamminedichloroplatinum(II), a more pronounced cell growth-inhibiting effect became evident: Application of 10^{-6} M *cis*-diamminedichloroplatinum(II) clearly reduced cellular multiplication to 22% of control values, a concentration of 10^{-5} M already induced a complete cessation of cellular proliferation.

Discussion

The findings of the present study show that some of the ferricenium complexes investigated exhibit antitumor efficiency against *solid* EAT *in vivo*. Because of the different site of tumor development and of substance injection, these results underline that the antineoplastic activity of ferricenium complexes is obviously based on a systemic effect. With respect to the pronounced tumor-inhibiting efficacy of the compounds, especially of **II** and **V**, against fluid EAT [5], however, the extent of reduction of tumor growth seems rather small just transcending the critical level of 50%. Other organometallic and inorganic cytostatic agents, such as titanocene dichloride and *cis*-diamminedichloroplatinum(II), are able to inhibit the growth of the same tumor in a much more pronounced manner resulting in a final tumor size of 14 or 19%, respectively [12].

On the other hand, the antiproliferative activity *in vitro* is stronger in the case of all ferricenium complexes investigated than for titanocene dichloride: Whereas the latter requires concentrations of 5×10^{-4} and 10^{-3} M to reduce significantly cellular proliferation [10], the ferricenium complexes already fulfil this effect in concentrations of 10^{-5} and 10^{-4} M. In the case of *cis*-diamminedichloroplatinum(II), an even lower concentration level (10^{-6} M) is sufficient for induction of an equivalent cytostatic action. This discrepancy concerning the *in vitro* cell growth-inhibiting activity of *cis*-diamminedichloroplatinum(II) on the one hand and of the metallocene compounds on the other hand may be explained by additional mechanisms occurring *in vivo* in the case of metallocenes, *e.g.*, by metabolic activation, by immunologic support or, simply, by a rapid hydrolytic degradation of metallocene compounds in aqueous solutions resulting in a diminution of cytostatic activity *in vitro*.

The high water solubility of ferricenium complexes is an obvious advantage with respect to their use in biological systems. Regarding, however, their reduced tumor-inhibiting activity against solid EAT shown in the present study as well as against leukemias L1210, P388 and B16 melanoma in comparison to titanocene dichloride (unpublished results of preliminary experiments), further antitumor testing experiments are necessary to decide about the tumor-inhibiting potential of ferricenium complexes.

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