Applied Organometallic Chemistry (1990) 4 19–26

0268-2605/90/010019-08/$05.00

Evaluation of the activity of some water-soluble ferrocene and ferricenium compounds against carcinoma of the lung by the human tumor clonogenic assay

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Received 16 August 1989 *Accepted 13 October 1989*

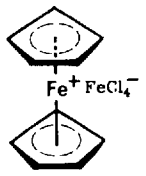
The two ferrocene compounds, ferrocenylacetic acid (3) and ferrocylthiomalic acid (4), as well as the ferricenium salts, ferricenium tetrachloroferrate (III) (1) and ferricenium trichloroacetate–trichloroacetic acid solvate (2), were investigated by the *in vitro* human tumor clonogenic assay for their inhibiting effects, in continuous exposure in a double-layer medium, on the colony formation of single-cell suspensions prepared from 30 selected fresh specimens of human adenocarcinoma, squamous cell carcinoma and large-cell carcinoma of the lung. At the high drug concentration level of 100 μg cm^{-3}, good response ratios were observed for 3 (79%) and 4 (72%), followed by 1 (67%) and 2 (52%). At the low concentration level of 10 μg cm^{-3} response ratios were below 25% for all compounds, best performance (24%) being shown by 1. *In vitro* experiments probing the schedule dependencies of the compounds' inhibiting effects against the lung carcinoma cell line PC-9 were also performed, as were experiments involving the combination of the ferricenium salt 1 and cisplatin, which showed the combination effect to be additive in both one-hour and continuous-exposure tests.

INTRODUCTION

The antineoplastic activity of certain ferricenium salts against Ehrlich ascites murine tumor lines (EAT), both liquid and solid, is on record.[1,2] Good-to-excellent cure rates were determined with the amply water-soluble salts **1** and **2**, ferricenium tetrachloroferrate (III) and ferricenium trichloroacetate–bis(trichloroacetic acid)-solvate, whereas low activity or inactive behavior was observed with poorly soluble or insoluble salts.

Once internalized into a particular body compartment, a ferrocene compound will exist as a ferricenium/ferrocene couple, the oxidation–reduction equilibrium position of which is dependent on the electrochemical conditions at that site and which can be approached both from the ferricenium and from the neutral ferrocene state. In certain biological environments, both oxidation and reduction reactions involving ferrocene or ferricenium species have been observed.[3–7] It may, therefore, prove irrelevant in the long run whether a bioactive agent containing the ferrocene complex system is administered in the ferrocene or in the ferricenium state, provided only that solubility, partition and diffusion properties of the administered form create favorable pharmacokinetics allowing for efficacious transport to the target tissue.

Against this background, the question arises as to whether antineoplastic activity might not perhaps be detectable with compounds administered in the uncharged (ferrocene) state. Ferrocene itself, which is practically insoluble in water, shows no *in vitro* or *in vivo* activity. In the present study we investigated the two hydrophilic and moderately water-soluble ferrocene compounds, ferrocenylacetic acid (**3**) and ferrocylthiomalic acid (**4**) (ferrocyl = ferrocenylmethyl),[8] in the human tumor clonogenic assay (HTCA) system. The water-soluble ferricenium salts **1** and **2**, both of which are active against EAT, were included for comparison.



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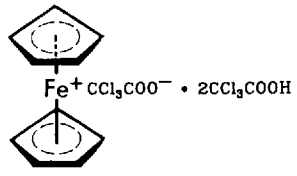

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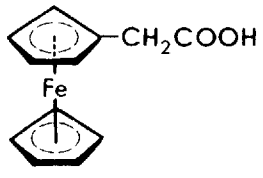

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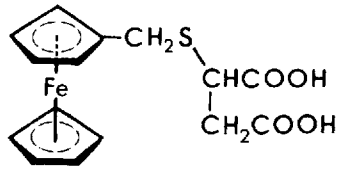

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The *in vitro* clonogenic assay, which exploits the capacity of human tumor cells for colony formation in double-layer soft agar, was introduced more than a decade ago by Hamburger and Salmon[9] as a useful predictor of the response of cancer patients to chemotherapy,[10–14] and Shoemaker *et al.*[15] addressed the feasibility of HTCA as a new system for antitumor drug screening. In particular, this system shows promise for screening novel agents with new chemical structures, and the use of cancer cells of human rather than rodent origin is desirable because experimental animal tumors may manifest drug sensitivities different from those of human tumor cells.

MATERIALS AND METHODS

Drugs

Cicplatin (*cis*-diamminedichloroplatinum(II)) was generously supplied by Nippon Kayaku Co., Limited, Tokyo, Japan. The ferricenium salts, ferricenium tetrachloroferrate(III) (**1**)[16] and ferricenium trichloroacetate–bis(trichloroacetic acid)-solvate (**2**),[17] were prepared as described.

Analysis: calcd for $C_{10}H_{10}Cl_4Fe_2$ (**1**): Cl, 37.0; Fe, 29.1; found: Cl, 37.2; Fe 29.0%. Calcd for $C_{16}H_{12}Cl_9FeO_6$ (**2**): Cl, 47.3; Fe, 8.3; found: Cl, 47.5; Fe, 8.0%.

Literature methods were also used for the synthesis of the uncharged compounds, ferrocenylacetic acid (**3**),[8,18] m.p. 165–166°C, and ferrocylthiomalic acid (**4**),[8] m.p. 174–176°C. (The term 'uncharged' refers to the oxidation state of the metallocence complex and does not relate to the dissociation of the carboxyl group in these compounds.)

Analysis: calcd for $C_{12}H_{12}FeO_2$ (**3**): Fe, 22.9; found: Fe, 22.7%. Calcd for $C_{15}H_{16}FeO_4S$ (**4**): Fe, 16.0; Found: Fe, 15.7%.

Patients' specimens

A total of 46 specimens, portions of which also served for an independent evaluation study of a new platinum compounds [*cis*-diammine(glycolato-*O,O'*)platinum(II); 254S],[14] were obtained from patients with carcinoma of the lung and were used in the present study. The tumor specimens were mechanically dissociated and suspended in McCoy's 5A medium containing 10% heat-inactivated fetal calf serum and penicillin/streptomycin solution (McCoy's wash; all from Grand Island Biological Co., Grand Island, NY, USA). Final tumor suspensions were prepared by passing the cells through a sterile stainless-steel screen (120–150 mesh). Viable nucleated cells were determined by Trypan Blue dye exclusion.[12]

Cell line

Human pulmonary adenocarcinoma cell line PC-9 derived from human adenocarcinoma of the lung (kindly provided by Professor Y. Hayata, Tokyo Medical College) was used in this study. The cells were propagated in RPMI 1640 medium supplemented with 10% fetal calf serum and streptomycin/penicillin solution in an incubator under a 5% CO_2 humidified atmosphere as described previously.[14]

In vitro drug sensitivity assessment

Utilizing a new drug screening program designed by the National Cancer Institute, Bethesda, MD, USA,[15] chemosensitivity was determined by continuous exposure of cells to drugs at the concentrations of 10 and 100 $\mu g\ cm^{-3}$ in a double-layer medium system over the entire culture period. Cells to be tested were suspended in top-layer CMRL 1066 medium supplemented with 15% horse serum and a variety of nutrients, and were then mixed with drug solution and Bacto-agar solution (final concentration 0.3%; DIFCO Lab., Detroit, MI, USA) in sequence. In the one-hour drug exposure experiment series, cells were treated with the test compounds in McCoy's wash for 1 h at 37°C. After drug exposure, the cells were washed twice with McCoy's wash, suspended in top-layer medium, and then mixed with agar solution. The resultant mixtures were pipetted in 1 cm^3 portions on to the bottom-layer medium, which consisted of 1 cm^3 of 0.5% agar in enriched McCoy's 5A medium, plated in 35 mm plastic Petri dishes. Numbers of cells plated were 5×10^5 and 1×10^4 cells/plate for fresh tumor cells and cell line PC-9, respectively. The plates were then incubated at 37°C in a 7.5% CO_2 high-humidity atmosphere. The number of colonies (>50 cells) was counted with a computerized image analyzer for cell line cultures on day 9, and with the aid of an inverted-phase microscope for primary tumor cultures on day 14. All counts were corrected for background colony counts by subtracting the colony counts on day 0. The percentage survival of tumor colonies was calculated by the equation:

$$\text{Colony survival (\%)} = \frac{\text{No. of colonies/plate (test culture)}}{\text{No. of colonies/plate (control)}} \times 100$$

A decrease of more than 50% in the percentage survival was defined as an *in vitro* response.

RESULTS

Inhibition of human tumor cell colony formation

In the first task, the inhibiting effect of the test compounds **1**–**4** on the colony formation of freshly isolated human tumor cells from 46 lung cancer patients was investigated by continuous exposure of the cells, in a double-layer medium, to each test compound over the entire culture period,[12] and counting for each culture the number of colonies per plate developed in the exposed medium. In 30 of these specimens, growth was sufficient (>30 colonies/plate) for assessing an antitumor activity at one concentration, at least, of the drug. These included 24 specimens from adenocarcinoma, five from squamous cell carcinoma, and a single one from large-cell carcinoma. In two parallel series of experiments, the test compounds were employed at concentration levels of 10 $\mu g\ cm^{-3}$ and 100 $\mu g\ cm^{-3}$, respectively. Examples of results obtained in two typical experiments are shown in Fig. 1(A) and (B). The percentage survival of tumor colonies is listed in Table 1 for the 30 specimens treated with **1**–**4**. The table also gives the response ratio, i.e. the number of *in vitro* responses per number of tests for each ferrocene derivative investigated. Of the four compounds tested, ferrocenylacetic acid (**3**) is seen to show the highest activity in the inhibition of colony formation. At the 100 $\mu g\ cm^{-3}$ level, the response ratio of this acid was 79%. This was closely followed by ferrocylthiomalic acid (**4**) with 72%. At the same concentration level, the ferricenium salts **1** and **2** gave response ratios of 67 and 52%, respectively. Note also that, in five cases of specimen resistance to both ferricenium salts, a response was exhibited by at least one of the two ferrocene compounds; Fig. 1(B) summarizes a representative case. These findings are significant insofar as the inhibiting effect of the two lower-oxidation-state ferrocene compounds **3** and **4** is shown to be as high as, if not higher than, that of the ferricenium salt **1**, whose superior potency against EAT is on record.[1] A much less impressive picture emerges in the test series run at the 10 $\mu g\ cm^{-3}$ concentration level, at which none of the four test compounds showed a particularly promising performance; i.e. response ratios remained below 25%. The ferricenium salt **1** now produced the highest activity among the compounds tested.

Schedule dependency of inhibitory effect

In order to assess the concentration and time dependencies of the inhibiting effects of **1**–**4**, two parallel series of experiments were conducted in which the lung carcinoma cell line PC-9 was exposed to the four compounds at concentration levels of 10, 25, 50, and 100 $\mu g\ cm^{-3}$. In the first series exposure was

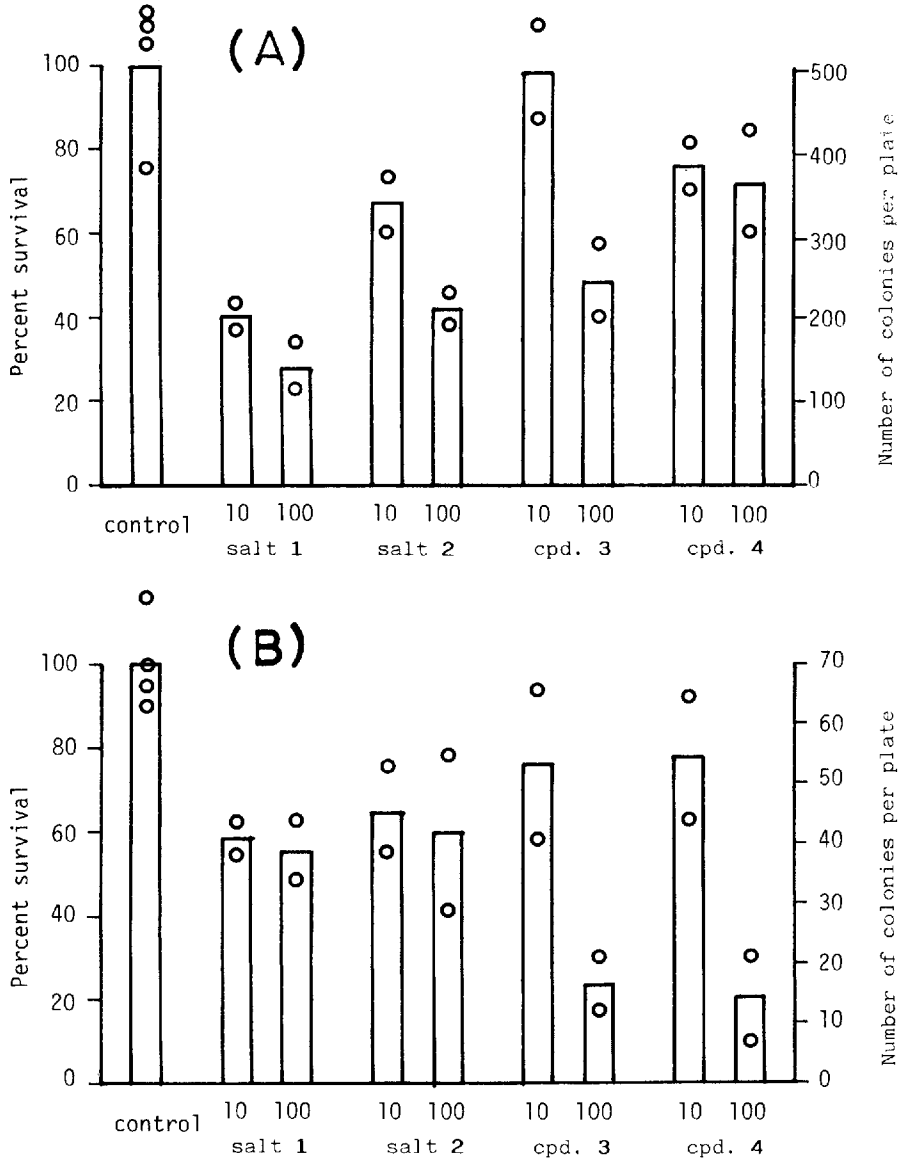

Figure 1 Colony formation of adenocarcinoma cells disaggregated from specimens of two lung cancer patients (A: 44, ♀; B: 65, ♂) and treated continuously with ferricenium and ferrocene complexes **1**–**4** (bars, percentage survival; o, number of colonies per plate). Tests were conducted in duplicate, at two drug concentrations (10 and 100 $\mu g\ cm^{-3}$), and control runs were performed in quadruplicate. Salt **1**, ferricenium tetrachloroferrate(III); salt **2**, ferricenium trichloroacetate — bis(trichloroacetic acid)-solvate; cpd **3**, ferrocenylacetic acid; cpd **4**, ferrocylthiomalic acid.

continuous, whereas in the second series it was restricted to 1 h. The results are given in Table 2. In the continuous-exposure series, the colony survival rate remained in the 75–100% range for all compounds up to 25 $\mu g\ cm^{-3}$, and for **2** and **4** up to 50 $\mu g\ cm^{-3}$. Ferrocenylacetic acid (**3**) gave the best performance, with colony survival down to 1.2 and 0.8% at 50 and 100 $\mu g\ cm^{-3}$ concentration levels, respectively. This was followed by the ferricenium salt **1**, which effected survival of 40 and

Table 1 Effects of ferricenium and ferrocene complexes **1**–**4** on colony formation of non-small cell lung carcinoma in human tumor clonogenic assay

Tumor specimens				No. of colonies per plate	Colony survival (%)[b]							
Patient	Age	Sex	Histology[a]		Ferricenium salt **1**		Ferricenium salt **2**		Ferrocene cpd **3**		Ferrocene cpd **4**	
					10 $\mu g\ cm^{-3}$	100 $\mu g\ cm^{-3}$ [c]	10 $\mu g\ cm^{-3}$	100 $\mu g\ cm^{-3}$	10 $\mu g\ cm^{-3}$	100 $\mu g\ cm^{-3}$	10 $\mu g\ cm^{-3}$	100 $\mu g\ cm^{-3}$
HK	72	M	AC	40	NT	6*	NT	20*	NT	0*	NT	0*
KT	44	F	AC	505	40*	28*	67	42*	76	72	98	48*
MS	73	M	AC	41	NT	41*	NT	69	NT	87	NT	54
MS	58	M	AC	65	89	76	105	94	NT	44*	NT	29*
SI	64	M	AC	39	NT	85	NT	NT	NT	NT	NT	NT
MT	42	F	AC	54	96	38*	72	90	99	39*	108	50*
KK	62	F	AC	77	36*	25*	82	48*	64	16*	100	33*
KH	56	M	AC	36	109	40*	47*	33*	40*	27*	61	42*
KF	50	M	AC	30	99	92	112	104	125	118	NT	101
KN	74	M	AC	120	77	29*	74	55	68	39*	57	24*
KO	68	M	AC	87	95	48*	54	24*	99	39*	96	35*
HK	81	M	AC	37	100	50*	58	40*	70	49*	84	49*
YT	53	M	AC	201	NT	96	NT	NT	NT	15*	NT	NT
YY	65	F	AC	54	79	69	90	85	93	50*	99	54
MO	45	M	AC	177	85	30*	82	22*	74	46*	74	76
KI	46	F	AC	133	45*	11*	77	49*	49*	0*	55	25*
AM	55	F	AC	70	58	55	64	60	78	20*	76	24*
RM	35	M	AC	101	NT	49*	NT	NT	NT	66	NT	NT
HT	60	M	AC	234	48*	22*	72	48*	70	11*	58	37*
SK	67	M	AC	55	92	43*	72	67	83	39*	68	66
HM	65	M	AC	42	125	92	84	100	93	75	102	39*
TA	64	M	AC	79	109	27*	64	37*	120	22*	88	47*
YN	49	M	AC	35	50*	20*	55	4*	11*	5*	36*	0*
TM	43	M	AC	151	67	42*	37*	11*	27*	13*	42*	26*
KT	72	M	SCC	35	NT	67	NT	80	NT	38*	NT	94
YK	65	M	SCC	36	NT	85	NT	NT	NT	43*	NT	NT
IH	66	M	SCC	33	NT	34*	NT	NT	NT	42*	NT	NT
SI	61	M	SCC	32	59	39*	86	49*	66	28*	48*	42*
TA	75	M	SCC	38	103	41*	104	77	80	75	95	42*
GS	68	M	LCC	84	NT	71	NT	90	NT	NT	NT	111
No. of responses/no. of tests (Response ratio (%))					5/21 (24)	20/30 (67)	2/21 (10)	13/25 (52)	4/20 (20)	22/28 (79)	3/19 (16)	18/25 (72)

[a] AC, adenocarcinoma; SCC, squamous cell carcinoma; LCC, large-cell carcinoma. [b] NT, not tested; * indicates response, defined as a decrease of 50% or more in the number of colonies per drug-treated plate relative to control plate. [c] Applied drug concentration.

Table 2 Inhibiting activity of ferricenium and ferrocene complexes **1**–**4** against colony formation of human lung cancer PC-9 cells in continuous-exposure and one-hour exposure systems

Compound		Continuous exposure			One-hour exposure		
Designation	Concentration ($\mu g\ cm^{-3}$)	No. of colonies per plate (± SD)[a]	Growth ratio[b] (%)	[IC_{50}]	No. of colonies per plate (± SD)[a]	Growth ratio[b] (%)	[IC_{50}]
Ferricenium salt **1**	0	2334 ± 84	—		2596 ± 118	—	
	10	2175 ± 98	93		2401 ± 106	92	
	25	1747 ± 108	75	[41]	1934 ± 50	74	[38]
	50	945 ± 60	40		930 ± 90	36	
	100	14 ± 5	0.6		39 ± 8	1.5	
Ferricenium salt **2**	0	2304 ± 149	—		2665 ± 113	—	
	10	2178 ± 118	95		2553 ± 85	96	
	25	2177 ± 153	94	[40]	2514 ± 74	94	[90]
	50	1973 ± 129	86		2102 ± 121	79	
	100	1434 ± 45	62		1210 ± 92	45	
Ferrocene cpd **3**	0	2376 ± 191	—		2635 ± 159	—	
	10	2087 ± 116	88		2643 ± 70	100	
	25	830 ± 62	85	[19]	2638 ± 58	100	Inactive
	50	28 ± 9	1.2		2614 ± 73	99	
	100	18 ± 7	0.8		2576 ± 117	98	
Ferrocene cpd **4**	0	2482 ± 151	—		2637 ± 59	—	
	10	2339 ± 193	94		2630 ± 85	100	
	25	2339 ± 100	94	[65]	2574 ± 19	98	Inactive
	50	2015 ± 146	81		2611 ± 89	99	
	100	22 ± 7	0.9		2582 ± 26	98	

[a] All determinations were performed in quadruplicate. [b] Ratio of number of colonies per drug-treated plate to number of colonies per control plate.

0.6% at these concentrations, and by ferrocylthiomalic acid, with, respectively, 81 and 0.9%. Poorest performance was delivered by the ferricenium salt **2**; colony survival with this compound exceeded 60% even at the highest concentration level (100 $\mu g\ cm^{-3}$). In the one-hour exposure series, the uncharged ferrocene compounds **3** and **4** remained inactive throughout. The ferricenium salts **1** and **2** caused survival of 1.5 and 45% at the 100 $\mu g\ cm^{-3}$ concentration, and of 36 and 79% at 50 $\mu g\ cm^{-3}$. Clearly, in the short-term exposure tests, superior performance of the ferricenium salts, notably the tetrachloroferrate **1**, is evidenced over that of the non-oxidized compounds.

Combination effect of ferricenium tetrachloroferrate (1) and cisplatin

In two series of tests, one utilizing continuous exposure, and the other, a one-hour exposure, the cell line PC-9 was treated with ferricenium tetrachloroferrate (**1**) and cisplatin both individually and in combination. The respective drug concentrations were equivalent to those which induced a 40% decrease in tumor colony-forming units of PC-9 cells, viz. 32 $\mu g\ cm^{-3}$ and 0.28 $\mu g\ cm^{-3}$, respectively. The percentage colony survival data are in Table 3, which also contains the percentage survival calculated for an additive effect of the two drugs in combination, by multiplying the survival fractions obtained for each drug in single application. It is seen that in both the short-term and the long-term exposure series, the observed and calculated values were almost coincident, indicating that the colony growth-inhibiting effects of the two compounds are additive.

DISCUSSION

In the human tumor clonogenic assay, tumor colony formation of carcinoma cells of the lung is inhibited

Table 3 Combination effects of ferricenium salt **1** with cisplatin on human lung cancer PC-9 cells in continuous-exposure and one-hour exposure systems of human tumour clonogenic assay

Compound	Continuous exposure		One-hour exposure	
Designation (Concentration[a] (μg cm^{-3}))	No. of colonies per plate ($\pm$ SD)[b]	Surviving fraction (%)	No. of colonies per plate ($\pm$ SD)[b]	Surviving fraction (%)
Control	2316 $\pm$ 65	100	2413 $\pm$ 40	100
Cisplatin (0.28)	1309 $\pm$ 91	56.5	1402 $\pm$ 48	59.6
1 (32)	1824 $\pm$ 81	78.8	1637 $\pm$ 45	69.5
Cisplatin (0.28) plus **1** (32)	1049 $\pm$ 57	45.3	981 $\pm$ 46	41.7
Expected surviving fraction in combination[c]		44.5		41.4

[a] Equivalent dose to 40% inhibition [IC_{40}] in tumor colony-forming units. [b] All determinations were performed six times. [c] Calculated by multiplying the surviving fraction resulting from cisplatin alone with that resulting from **1** alone.

not only by the ferricenium salts **1** and **2**, whose activity against EAT had previously been established, but also, albeit in a different concentration–time dependency, by the uncharged ferrocene compounds **3** and **4**. Future work is needed to address the question as to whether the neutral ferrocene compounds are acting as prodrugs and may need an incubation time for oxidation to the ferricenium state in order to develop their potential as *in vitro* inhibitors of colony formation.

In assessing the overall potency of the compounds, one must recognize that the predictive accuracy of the clonogenic assay is optimized at *in vitro* drug dosage concentrations approximating 10% of the respective peak plasma concentrations (PPC) of the drug attainable with standard *in vivo* drug doses.[9]

The PPC values of compounds **1**–**4** are not known. For the ferricenium salts **1** and **2**, however, LD_{50} data are available (240 and 400 mg kg^{-1}, respectively[1]), and the PPC values can be assessed from these with the aid of the relationship:[19]

$$\log (\text{PPC}) = -0.788 + [0.755 \times \log(LD_{50})]$$

Peak concentrations of 10 and 15 μg cm^{-3}, respectively, are thus calculated for **1** and **2**. It follows that, at least for the ferricenium salts, the drug concentrations employed in the HTCA should, for optimal utilization of the assay, be below the 10 μg cm^{-3} level used here, and it is clear that at such low drug concentrations response ratios considerably lower even than tabulated for the 10 μg cm^{-3} series must be expected. For the two compounds **3** and **4** no LD_{50} data exist; hence, no PPC assessments are available. We anticipate somewhat decreased toxicity for these uncharged compounds relative to the oxidized complexes (toxicology work is in progress), and this should, with regard to the colony growth inhibition effect, place a higher premium on **3** and **4** than on **1** and **2**.

Despite the obvious limitations imposed by the high doses required to elicit satisfactory response ratios, the results are considered promising enough to warrant an extension of this work with the aim of evaluating a larger number of water-soluble ferrocene compounds and their oxidized ferricenium counterparts with respect to their toxicological behavior and their inhibiting activity in the clonogenic assay.

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