Cytotoxicity of a Series of Ferrocene-containing β-Diketones

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Abstract. *Background: Oxidised ferrocenium compounds often possess antineoplastic activity. In contrast, reduced ferrocene derivatives frequently only show activity if cell components can oxidise them inside cells to the ferrocenium species. Ferrocene compounds having the lowest formal reduction potential are normally expected to be the most cytotoxic. Here we demonstrate this is not always the case. Some of the structure-related and physical properties that enhance ferrocenyl antineoplastic activity have been investigated. Materials and Methods: Ferrocene-containing β-diketones of the type $FcCOCH_2COR$ with Fc=ferrocenyl and R=CF_3, CCl_3, CH_3, Ph(=C_6H_5, phenyl) and Fc, were tested for cytotoxicity against HeLa (human cervix epitheloid), COR L23 (human large cell lung carcinoma) and platinum resistant CoLo320DM (human colorectal) and COR L23/CPR cancer cell lines. Cell survival was measured by means of the colorometric 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide (MTT) assay. Results: The mean drug concentration from 3 experiments causing 50% cell growth inhibition, (IC_{50}) values, varied between 4.5 and 85.0 μmol dm^{-3}, with the CF_3-containing β-diketone being the most active. Drug activity was inversely proportional to the formal reduction potential, $E^{o'}$, of the ferrocenyl group, and dependent on the R group in the general β-diketone structure. The CF_3 complex was more cytotocic than cisplatin inter alia against platinum-resistant cell lines, and at least eight times more reactive against cancer cell lines than against PHA (phytohaemagglutinin)-stimulated lymphocyte cultures. Conclusion: A drug activity–structural relationship exists in that ferrocenyl drugs with halogen substituents chains are more cytotoxic. Compounds with higher ferrocenyl group formal reduction potential and stronger acid strength (i.e. smaller pK_a value) are more cytotoxic.*

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Often potentially good chemotherapeutic drugs find limited clinical use due to the many negative medical and physical side-effects they exhibit. For cisplatin, ($Pt(NH)_3Cl_2$), these include *inter alia* poor aqueous solubility, a high excretion rate from the body (1), loss of appetite (anorexia) (2), development of drug resistance after continued drug dosage (3), high toxicity especially to the kidneys and bone marrow (4), and, perhaps most important of all, inability to distinguish between healthy and carcinomatous cells (5). To combat these negative side-effects of chemotherapeutic drugs, new antineoplastic materials are continuously being synthesised and evaluated, combination therapy has been investigated in the hope of finding synergistic effects (6), new methods of delivering an active drug to a malignant growth are being developed (7, 8) and new techniques of cancer treatment, such as photodynamic cancer therapy (9), are being investigated.

In terms of new antineoplastic material, it has been shown that certain ferricenium salts (10) had more favourable 50% lethal dosage values than cisplatin (11), while water-soluble ferrocene-containing carboxylates (12) induced good to excellent cure rates against human adenocarcinoma, squamous cell carcinoma and large-cell carcinoma of the lung in *in vitro* human tumour clonogenic assays. It has also been shown (8) that by anchoring the antineoplastic ferrocene derivative 3-ferrocenylbutanoic acid on a water-soluble polymeric drug carrier, an increase in drug activity of almost one order of magnitude may be obtained.

The mechanism by which the ferrocenyl group destroys antineoplastic growth was shown to involve homolytic action, *i.e.* radical-induced electron transfer processes (13) between a ferrocenium group and water, *inter alia,* to generate hydroxy radicals which cleave DNA strands. This implies a ferrocene-containing drug must, after it is administered to the body, first be oxidised to the ferrocenium species to show antineoplastic activity. The redox chemistry of the ferrocenyl moiety is not

complex, as the iron core of the ferrocenyl group in the reduced Fe^{II} state (the neutral ferrocenyl state), or the oxidised Fe^{III} state (the cationic ferrocenium state) have the same co-ordination sphere. Electron transfer processes between the ferrocenyl and ferrocenium states are fast and reversible. Once internalised into a particular body compartment, the ferrocenyl moiety of a compound will exist as a mixture of the neutral ferrocenyl and cationic radical ferrocenium species (14-18). The equilibrium position is dependent on the electrochemical conditions in the particular body compartment. It is, therefore, irrelevant whether the bioactive agent containing the ferrocenyl group is administered in the reduced ferrocenyl or oxidised ferrocenium state provided the formal reduction potential of the ferrocenyl group is low enough to allow ferrocenyl oxidation inside a cell. Indications are that the cut-off potential is ~ +0.2 V *vs*. SCE (saturated calomel reference electrode) or 0.52 V *vs*. Fc/Fc^+ where Fc=ferrocenyl (8). In support of this conclusion, it was recently shown that a series of ferrocene-containing alcohols do show enhanced antineoplastic activity (19). The compounds became more cytotoxic the lower the formal reduction potential of the ferrocenyl group became.

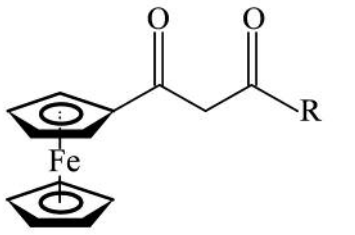
Towards the goal of improved drug performance against malignant cells, the results of *in vitro* cytotoxicity tests of a series of ferrocene-containing β-diketones **1-5** (Figure 1) are here reported and the beneficial effect halogenated groups may have on drug activity is demonstrated. The results are also correlated with the formal reduction potentials (20) of the ferrocenyl moiety and the acid strength (pK_a values) of all the investigated β-diketones.

Materials and Methods

Compounds. β-Diketones **1-5** (Figure 1) were synthesised according to published procedures (21).

Sample preparation. The samples were dissolved in dimethyl sulphoxide (DMSO) giving stock concentrations of 20 mmol dm^{-3} and diluted in the appropriate growth medium supplemented with fetal calf serum (FCS) to give final DMSO concentrations not exceeding 0.5% and drug concentrations of 20-2,000 μmol dm^{-3} prior to the cell experiments.

Cell cultures. The human colorectal cell line, CoLo 320DM (ATCC CCL-220) (American Type Culture Collection, Manassas, Virginia, USA), was grown as a suspended culture in RPMI-1640. The human cervix epitheloid cancer cell line, HeLa (ATCC CCL-2) (American Type Culture Collection), human large-cell lung carcinoma cell line, COR-L23 (ECACC 92031919) (European Collection of Cell Cultures, Salisbury, Witshire, UK) and a cisplatin-resistant sub-line, COR-L23/CPR (ECACC96042336) (European Collection of Cell Cultures), were grown as monolayer cultures using MEM in the case of HeLa cells and RPMI-1640 in the case of the human large-cell lung carcinoma cell lines. The growth media were maintained at 37˚C under 5% CO_2 and fortified with 10% FCS, and 1% penicillin and streptomycin.



1: R = CF_3, 2: R = CCl_3, 3: R = CH_3, 4: R = Ph, 5: R = Fc

Figure 1. *Structure of ferrocene-containing β-diketones $FcCOCH_2COR$. Ph=phenyl=C_6H_5, Fc=ferrocenyl=$(C_5H_5)Fe(C_5H_4)$.*

Purified mononuclear leukocytes were prepared from whole blood collected from healthy donors by density centrifugation on Histopague-1077 (Sigma-Aldrich, St Louis, Missouri, USA) cushions at 400xg for 25 minutes at room temperature. The lymphocyte band was washed and cells resuspended in RPMI-1640 supplemented with 10% FCS. Cells were seeded (at 400 cells/well in the case of cancer cells and 2×10^3 cells/well in the case of mononuclear leukocytes) in 96 well microtiter plates in a final volume of 200 μl of growth medium in the presence or absence of different concentrations of experimental drugs. Appropriate solvent control systems were included. To some of the wells, a mitogen (phytohaemagglutinin, PHA; Remel Europe Ltd, Dartford, Kent, UK) was added at a concentration of 2.5 μg/ml. After incubation at 37˚C for 7 days in the case of cancer cells and 3 days in the case of the mononuclear leukocytes, cell survival was measured by means of the colorometric 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide (MTT) assay (8, 22, 23). Wells without cells and with cells but without drugs were included as controls. Survival curves were plotted (Figure 2) as a function of drug dose and IC_{50} (drug concentration that causes 50% inhibition of cell growth) were estimated by extrapolation.

Results and Discussion

Since it is known that ferrocene-based drugs often need an incubation period before they perform optimally in cell destruction, all the studies utilising β-diketones **1-5** were performed utilising seven days of drug exposure to the cells in accordance with previous studies (8, 19). The cell growth inhibitory properties of ferrocene β-diketone derivatives **1-5** expressed as IC_{50} values are summarised in Table I. The lowest IC_{50} values correspond to the more active compounds. The most active drug was found to be the CF_3 complex **1**. For **1**, cell growth inhibition of COR L23 cells exceeding 95% (not to be confused with IC_{50} values of Table I) were observed at 12.5 μmol dm^{-3} concentrations, Figure 2. The least sensitive cell line to the CF_3 complex **1** was the HeLa cell line. In this case, a concentration of 100 μmol dm^{-3} of **1** was needed to achieve 95% inhibition of the growth of HeLa cells. Ferrocene complexes **1-5** also showed good activity against CoLo 320DM, an intrinsically multidrug-resistant human colon adenocarcinoma cell line, and Cor L23/CPR, a

Table I. *Chemosensitivity of β-diketones **1-5** and cisplatin expressed as IC_{50} (μmol dm^{-3}) values[a] after 7 days of incubation with the indicated cancer cell lines or 3 days of incubation with PHA-stimulated human mononuclear leukocytes (SHML). Formal reduction potentials, $E^{o'}$, of the ferrocenyl group are vs. a Fc/Fc^+ reference electrode. Values in brackets are the quotient (IC_{50} of SHML)/(IC_{50} of a particular cancer cell line). pK_a=acid strength, nd=not determined.*

Compound	$E^{o'}$ (V)[b]	pK_a[c] L23	HeLa L23/CPR	COR 320DM	COR	CoLo	SHML
1 (R=CF_3)	0.317	7.15	6.8 (12.2)	4.5 (18.5)	6.3 (13.2)	7.3 (11.4)	83.1
2 (R=CCl_3)	0.293	7.65	37.7 (1.8)	12.5 (5.4)	20.1 (3.4)	28.4 (2.4)	67.5
3 (R=CH_3)	0.236	10.01	66.6 (-)	nd (-)	nd (-)	57.1 (-)	nd
4 (R=Ph)	0.230	10.41	54.2 (-)	66.8 (-)	80.4 (-)	85.1 (-)	>100
5 (R=Fc)	0.187d	13.10	54.4 (-)	75.4 (-)	74.4 (-)	64.3 (-)	>100
Cisplatin	-	-	5.6 (6.9)	6.3 (6.1)	12.3 (3.1)	13.0 (3.0)	38.4

[a]Data from three experiments are expressed as the mean drug concentration (μmol dm^{-3}) causing 50% inhibition of cell growth; [b]Data from ref 20; [c]Data from ref 21; [d]The first formal reduction potential of this diferrocene-containing compound. The second formal reduction potential is at 0.297 V.

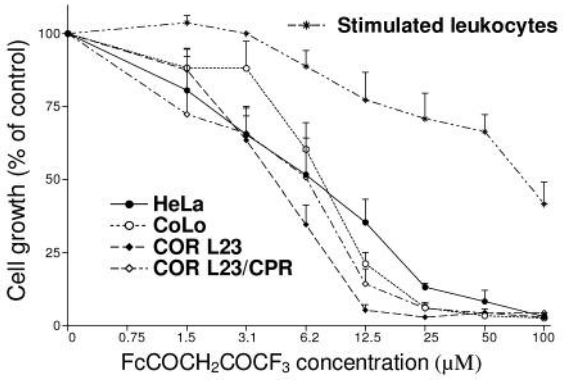


Figure 2. *Effect of β-diketone **1** on the survival of various human cancer cell lines and PHA-stimulated mononuclear leukocytes after 7 (or 3 for SHML) days of incubation measured as a percentage of untreated controls. Each end-point represents the mean of three experiments ± standard error of the mean.*

varient of COR L23 resistant to malphalan and other platinum compounds. Figure 2 illustrates that CF_3 complex, **1,** achieved more than 95% inhibition of cell growth for these two cell lines at concentrations of 50 μM or even less. The halogenated complexes **1** and **2** were much more active than the halogen-free complexes **3** and **4,** and the diferrocenylated complex **5**, indicating that enhanced cell growth inhibitory effects may be achieved by incorporating halogens in antineoplastic drugs.

For PHA-stimulated lymphocyte cultures, the ferrocene complexes tested led to 50% inhibition of cell growth only at concentrations of 67.5 μmol dm^{-3} and higher (Table I). The ideal antitumour agent should be an agent with high activity against cancer cells, including drug-resistant cells, and low activity against stimulated mononuclear leukocytes. The CF_3 complex, **1,** possessed the highest tumour specificity and from the quotients shown in Table I, depending on the cancer cell line, was eleven to eighteen times less active against normal human mononuclear leukocytes than tumour cells. The same quotient for cisplatin varied between 3.0 and 6.9, indicating that β-diketone **1** was much more selective in decreasing cell growth in cancer cells.

Previous studies by us as well as others indicated that ferrocene derivatives appear only to possess reasonable antineoplastic activity if the formal reduction potential of the ferrocenyl group is 0.21 V or less *vs*. SCE (8), or 0.03 V *vs*. a Ag/Ag^+ reference electrode (13, 19). Due to the toxicity of mercury, the use of calomel reference electrode systems has fallen into disfavour. To convert SCE or Ag/Ag^+ referenced potentials to Fc/Fc^+ referenced potentials, the newest IUPAC reference potential, subtract 0.39 or 0.08 V respectively as described elsewhere (24). In this study, all the β-diketones have formal reduction potentials less than 0.317 V *vs*. Fc/Fc^+. The most active compound, complex **1**, has a formal reduction potential of 0.317 V *vs*. Fc/Fc^+ (Table I), while the least active complexes **4** and **5** have much smaller formal reduction potentials, 0.230 and 0.187 V respectively. This was in sharp contrast with previous research on ferrocene-containing amides (8) and alcohols (19), which indicated that compounds with lower reduction potentials should be more cytotoxic. Based on formal reduction potentials only, one would expect complex **5** to be the most active, not the least active, as it has the smallest reduction potential of all the compounds investigated (Table I).

Evidently, the redox potential of the ferrocenyl group is not the only point that determines the antineoplastic activity of a ferrocene compound. Structural features of ferrocene derivatives, such as substituent chain length (8), have been shown to be an important parameter in cytotoxicity. For the present β-diketone series, the planar shape of the β-diketones in the enol form (20) may be important. The relative acidity, expressed as pK_a values, of complexes **1-5** is another important variable that will determine the antineoplastic activity of ferrocene complexes. The

two most effective compounds tested here, **1** and **2**, are by far the strongest acids, having pK_a of 6.53 and 7.15 respectively (20). The pK_a values listed in Table I imply that only these two complexes would exist as the diketonato salts at normal blood pH of 8. All the other β-diketones are so basic that they would remain in the neutral β-diketone form at blood pH, and this would impair aqueous solubility.

Conclusion

Of the five ferrocene-containing β-diketones that were tested for antineoplastic activity against human cancer cell lines, the trifluoro-containing β-diketone, **1,** was the most active, despite having the largest ferrocenyl formal reduction potential. Higher activity for ferrocene-containing β-diketones is associated with stronger acid strength, *i.e.* lower pK_a values. Compound **1** shows appreciable activity against platinum-resistant cancer cell lines and is two to three times more selective in decreasing cell growth than cisplatin. This class of compounds merits further investigation as potential chemotherapeutic drugs.

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