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Antineoplastic Activity of Polyaspartamide–Ferrocene Conjugates†

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The ferrocene/ferricenium redox system plays a significant role in biological oxidation, reduction and free-radical reactions. Of particular interest are the findings of earlier investigations which showed certain water-soluble ferricenium salts to possess appreciable antiproliferative activity against various murine tumor lines and a xenografted human colorectal adenocarcinoma. Solubility in water, a prerequisite for efficacious transport and dissipation in central circulation, was then proposed as a principal requirement for the ferrocene complex system to exert antineoplastic activity irrespective of the oxidation state in which it is administered. In order to shed more light on this question, we decided to investigate the antiproliferative properties of polymer–ferrocene conjugates containing the metal complex in the non-oxidized (ferrocene) form while fulfilling the critical requirement of water solubility. To this end, five selected, water-soluble conjugates, synthesized by reversible coupling of 4-ferrocenylbutanoic acid to variously structured polyaspartamides featuring pendant primary amino groups as coupling sites, were tested *in vitro* against cultured HeLa cells at concentrations up to 50 μg Fe ml^{-1}. Optimal antiproliferative activities, with IC_{50} in the range of 2–7 μg Fe ml^{-1}, were determined for three compounds possessing tertiary-amine functions susceptible to protonation at physiological pH. Lower activities (IC_{50} = 45–60 μg Fe ml^{-1}) were demonstrated for two poly(ethylene oxide)-containing conjugates. However, no reasonable structure–performance relationships can be derived at this stage from the small number of compounds tested. © **1998 John Wiley & Sons, Ltd.**

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INTRODUCTION

As a metallocene of the transition-metal series, the di-η^5-cyclopentadienyliron(II) compound, ferrocene (**1**), shows an unusual oxidation/reduction behavior. The neutral, uncharged compound readily loses an electron, thereby converting to its one-electron oxidation product, the ferricenium cation ($\mathbf{1}^+$) (Scheme [1]). Holding an unpaired electron in one of the two non-bonding e_{2g} orbitals, the cation represents a free-radical species of appreciable stability.[1] With free-radical processes abundant in the biological world, it is not surprising that the ferrocene/ferricenium system has become a study object *par excellence* for researchers in the biochemical and biomedical disciplines. The ferricenium cation is biologically accessible to enzymically mediated oxidation of **1** by hydrogen peroxide.[2] The reverse reaction, $\mathbf{1}^+ \rightarrow \mathbf{1}$, is mediated by NADH[3] and metalloproteins;[4] in addition, $\mathbf{1}^+$ undergoes recombination reactions with other free radicals, which, after proton elimination, leads to substituted, uncharged ferrocene compounds.[1] The cation is also reduced by interaction with the biologically important superoxide anion radical, regenerating dioxygen in the process.[5] Another biologically significant reaction step involves oxidation of ferrocenylcarboxylate anion by the notoriously aggressive hydroxyl radical, affording

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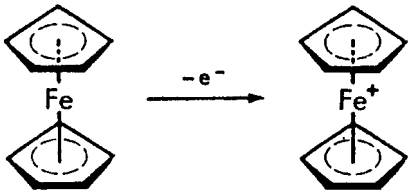
ferriceniumcarboxylate in addition to the (biologically harmless) hydroxyl anion.[6]



1 **1⁺**

The role of free radicals in the initiation, progression and control of cancerous lesions has been a focal point of interest in cancer research for many years,[7] and the peculiar redox behavior of the ferrocene system prompted the early[8,9] proposal that polymeric ferrocenes should be investigated for charge-transfer interactions in carcinogenic processes. The various subsequently reported free-radical and superoxide-scavenging reaction sequences discussed in the preceding paragraph suggest that the **1**/**1**$^+$ system could indeed have a part to play in cancer prevention or inhibition. In order to explore this question more tangibly, we reinvestigated numerous ferricenium salts and other ferrocene derivatives for the purpose of providing pure and compositionally well-defined compounds for biomedical evaluation.[10–17] These included, *inter alia*, the ferricenium tri- and penta-iodides,[11,12] the trichloroacetates,[10,11,15] the tetrachloroferrate and some μ-oxodiferrates.[11,13,14] Furthermore, collaborative testing programs were initiated with biomedical research groups, in which a number of ferricenium and ferrocene compounds were screeened for activity against ascitic murine tumors[18–20] and human clonogenic cancer lines.[21] The findings, demonstrating the need for solubility in aqueous media as a means of rapid distribution in central circulation, showed lack of activity for water-insoluble ferricenium salts but a high degree of activity for a number of readily soluble ferricenium salts, cure rates in two instances attaining the 100% level against Ehrlich ascites.[18] Activity was later observed also against Colon 38 adenocarcinoma, Lewis Lung carcinosarcoma, and a xenografted human colorectal carcinoma.[22] Promising activity behavior was reported independently for the tetrachloroferrate[23] and for the triiodide,[24] with therapeutic indices appreciably larger than determined for *cisplatin.* Lastly, activity was shown by certain azole derivaties of ferrocene which could potentially exist in a form featuring a cationic site.[25] As the clonogenic assay[21] revealed some activity even for the two non-oxidized but moderately water-soluble compounds, ferrocenylacetic acid and ferrocylthiomalic acid, whereas an equally non-oxidized but water-insoluble ferrocenyl-substituted *cis*-diaminedichloroplatinum(II) complex proved inactive,[26] we concluded[27,28] that it should be irrelevant whether a ferrocene compound be administered in the original (ferrocene) or in the oxidized (ferricenium) state, as long as the compound possessed sufficient water solubility to enter the vascular system rapidly. The **1**/**1**$^+$ equilibrium distribution in any body compartment should, after all, be dictated in the first place by the compound's reduction potential and by such environmental conditions as pH or enzymic activity, rather than by the oxidation state in which the compound existed when originally administered. These considerations prompted us to design and synthesize macromolecular ferrocene compounds in which the inherently hydrophobic and water-insoluble metallocene unit is reversibly attached to polymeric carriers possessing complete solubility in water, thus providing a vehicle for efficacious intravascular dissipation.[27–30] The potential for enhanced chemotherapeutic effectiveness of drug species conjugated to polymeric carriers has been extensively investigated and reviewed in the past two decades, most recently by Duncan[31] and by Putnam and Kopeček.[32] In the present communication we report on preliminary screening *in vitro* of selected water-soluble macromolecular ferrocene conjugates for antiproliferative activity against the human HeLa cancer line.

RESULTS AND DISCUSSION

The conjugates selected for activity screening, **2–6,** possessed the structures shown in Figure 1.

Conjugates **2, 4** and **5** are known compounds, prepared in a preceding investigation by coupling of 4-ferrocenylbutanoic acid to the primary amine side functions of presynthesized polyaspartamide carriers.[33] Conjugate **3** was obtained by an analogous procedure involving coupling of the same butanoic acid to the known carrier, poly-α,β-D,L-[*N*-(3-(dimethylamino)propyl)aspartamide(75)-*co*-*N*-(3-aminopropyl)aspartamide-(25)].[34] For the preparation of conjugate **6,** a polyaspartamide carrier was prepared by the procedure developed in a previous study for the

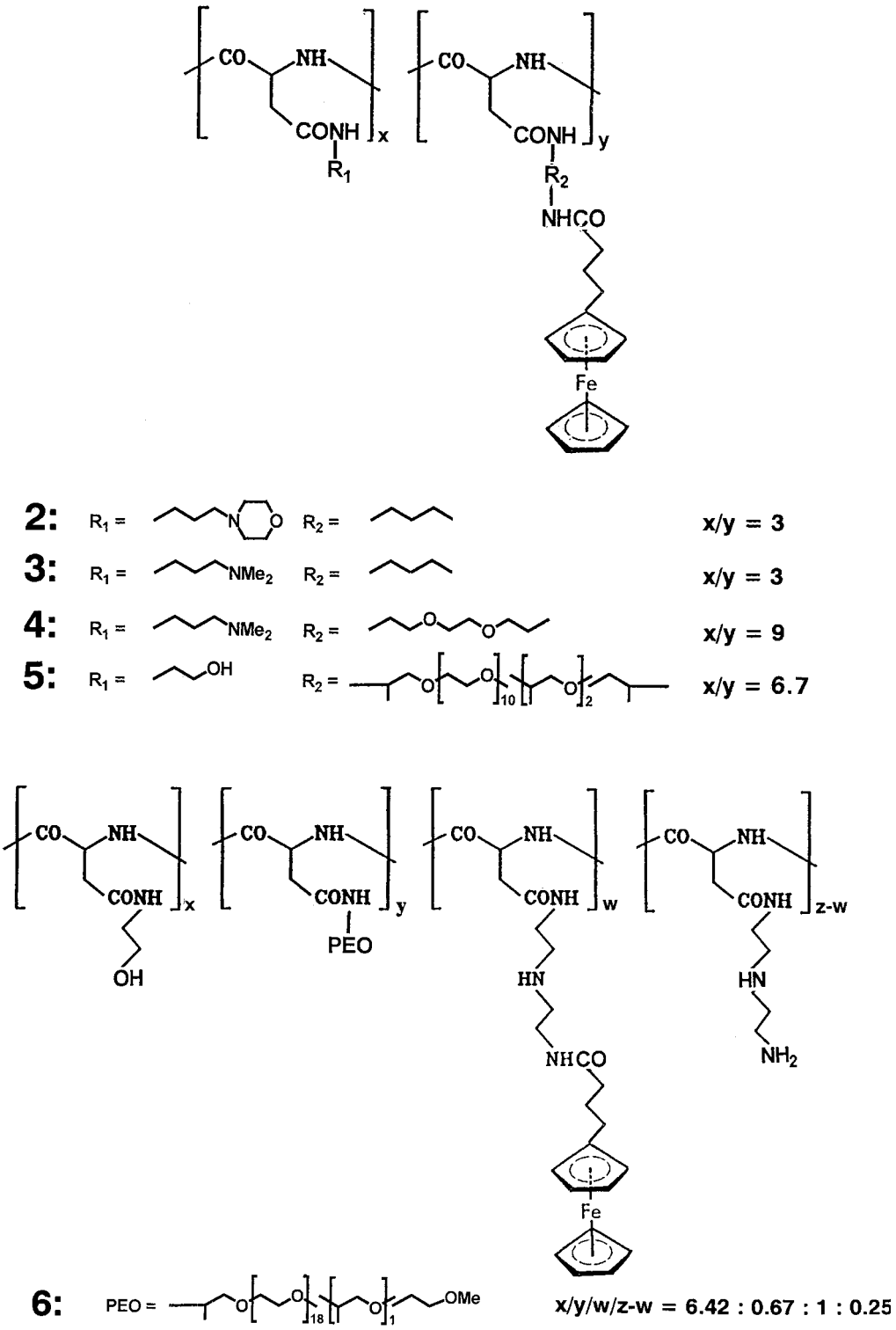


Figure 1 Structures **2–6.**

synthesis of similar polyamides.[35] This involved the stepwise treatment of poly-D, L-succinimide in *N*,*N*-dimethylformamide solution with given amounts of the amine nucleophiles Jeffamine M-1000, diethylenetriamine and ethanolamine, in that order. The resulting copolyaspartamide was separated as in the preceding work.[35] The water-soluble polymer contained poly(ethylene oxide)-modified units (8 mol%), ethylenediamine-modified units (15 mol%) and hydroxylethyl-modified units (77 mol%), as found by ^{1}H NMR. Coupling of 4-ferrocenylbutanoic acid to the carrier so obtained gave conjugate **6,** in which 80% of available NH_2 groups were ferrocenylated. All product polymers were fractionated by dialysis for removal of material with molecular masses substantially below 25 000 in order to retard renal clearance. The choice of the 4-butanoic acid derivative of ferrocene as the active agent in this study was based on its low formal reduction potential ($E^{\circ\prime}$ = 0.172 V vs SCE; in aqueous ethanol) relative to ferrocene (0.199 V) and other ferrocenylcarboxylic acids,[30] thus ensuring ease of oxidation in the biological environment. The peptidic main-chain compositions were chosen for reasons of biocompatibility and ease of endocytotic tumor-cell entry prompted by the elevated amino acid demand of the transformed cell. The chains, all of the α,β-D,L-configurated type, are expected to be comparatively stable while in circulation, the presence of β-peptide and D-configured units preventing premature degradation by enzymic (α-peptidase) 'unzipping' action; upon cell entry, however, lysosomal and hydrolytic (pH reduced to approx. 5) action should cause fragmentation and ultimate cleavage of all amide links, including those of the spacers conjugating the ferrocenylbutanoic acid, thus liberating the ferrocene compound for interaction with the nuclear material.

The compounds were tested for activity against cultured HeLa cells over a period of 72 h at concentrations of up to 50 μg Fe ml^{-1}. The results in terms of percentage cell growth (relative to control) versus concentration are plotted in Figure 2. IC_{50} data (μg Fe ml^{-1}) are presented in Table 1, which also lists the percentage Fe content of the conjugates. Best performance is indicated for **3,** closely followed by **4,** with IC_{50} in the vicinity of 2 μg ml^{-1}. A slightly higher value (7 μg ml^{-1}) is apparent for **2.** Significantly lower activites are shown by the remaining two compounds **5** and **6,** with IC_{50} values of 45 and (extrapolated) 60 μg ml^{-1}, respectively. A common feature of the first-named three conjugates is the presence of a predominant proportion of tertiary-amine side functions susceptible to protonation at physiological pH. Facilitated cell entry through adsorptive pinocytosis has been observed with cationic polymers, such as polylysine.[36–38] Furthermore, certain types of cancer cell develop a negative surface charge and are thus susceptible to preferential approach by cationic substrates.[39] Whether the potentially cationic side groups in **2**–**4** do indeed play an active role in facilitating the pinocytotic uptake by the transformed cell remains an open question. Conjugates **5** and **6** both contain comparatively long poly(ethylene oxide) side chains, introduced here to increase hydrophilicity and reduce immunogenicity and protein binding.[40] The inferior performance of the two compounds might be associated with these structural features. The coiling poly(ethylene oxide) chains may cause diminished steric accessibility of the spacer-incorporated amide links to lysosomal enzymes with consequently decreased metallocene bioavailability. Other observations,[41] however, seem to invalidate this argument. Although the preliminary activity data presented here confirm our earlier expectations, a considerably larger number of exemplifying polymer structures possessing a wide range of side groups and spacer attachment designs will have to be investigated in an effort to establish meaningful structure–performance relationships that may serve as a basis for further development work.

Table 1 Activity of conjugates **2–6** against cultured HeLa cells

Conjugate	Fe in conjugate (%) Found	Calcd.	IC_{50}[a] (μg/Fe ml^{-1})
2	5.03	4.86	7.2
3	5.50	5.46	2.1
4	2.35	2.44	2.3
5	3.45	3.34	45
6	2.25	2.49	60[b]

[a] Mean Fe concentration (3 experiments) causing 50% inhibition of cell growth.
[b] Approximated by extrapolation to concentration >50 μg ml^{-1}.

EXPERIMENTAL

Conjugates

Amounts of polymeric compounds are given as

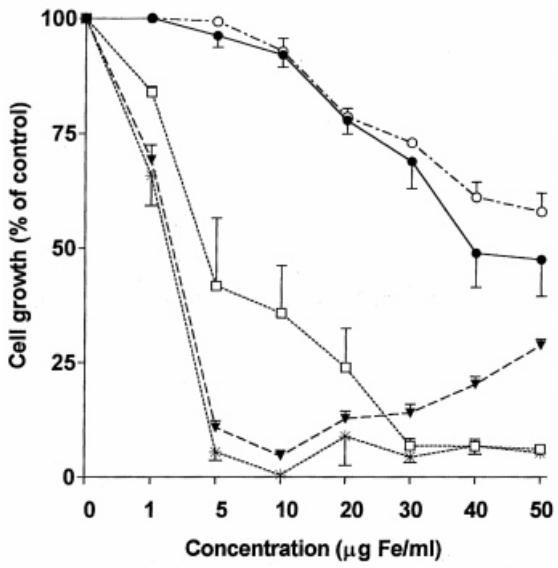


Figure 2 Percentage cell growth, relative to control, versus concentation (μg Fe ml^{-1}). ∗ **3;** ▼ **4;** □ **2;** ● **5;** ○ **6**.

base moles and, thus, refer to the simplest recurring units, defined by structures **2**–**5** normalized to $y = 1$, and by structure **6** normalized to $w = 1$. The compounds **2, 4** and **5,** prepared and characterized in the preceding investigation[33] (there designated as **1–Fc, 4**(90:10)–**Fc,** and **5**(87:13)– **Fc,** respectively, were used as received from that study.

For the synthesis of conjugate **3,** not heretofore described, a solution was prepared from the carrier, poly-α,β-D,L-[*N*-(3-(dimethylamino)propyl)aspartamide(75)-*co*-*N*-(3-aminopropyl)aspartamide(25)[34] (154 mg; 0.2 mmol), in 3 ml of dry, freshly distilled *N*,*N*-dimethylformamide. Upon the addition of triethylamine (61 mg; 0.6 mmol) and *N*-succinimidyl 4-ferrocenylbutanoate (133 mg; 0.36 mmol), the solution was saturated with nitrogen and stirred in the stoppered flask for 48 h at ambient temperature and for another 8 h at 65 °C. The pH at this point was approx. 8. The product polymer was precipitated from the cooled solution with excess Et_2O, washed with hexane, dissolved in 30 ml of H_2O and dialyzed in Spectra/Por 4 tubing (molecular mass cut-off 12 000–14 000; Spectrum Industries, Los Angeles, CA, USA) for 0.5 h in H_2O at pH 8 (Na_2CO_3), followed by dialysis in Spectra/Por 6 tubing (molecular mass cut-off 25 000) for 20 h against deionized H_2O containing ascorbic acid (20 mg), then for 3 h against plain H_2O. The retentate, on freeze-drying, gave **3** as a tan-colored solid (111 mg; 54.2%); η_{inh} (H_2O; $c = 0.2$ g/100 ml), 8 ml g^{-1}. ^{1}H NMR (D_2O, pH 10)δ (expected proton count in parentheses): 4.2–4.1 ppm, 9H (9H; cyclopentadienyl); 1.8–1.6 ppm, 10H (10H; $CH_2CH_2CH_2$). Analysis: calcd for $(C_{48}H_{78}FeN_{12}O_9)_n$ (1023)$_n$ (**3**): C, 56.35; H, 7.69; N, 16.43; Fe, 5.46%; C/N, 4:1. Found: C, 55.17, H, 7.52, N, 15.78; Fe, 5.50%; C/N, 4.08:1.

The carrier required for the preparation of conjugate **6** was synthesized as described below. The poly(ethylene oxide) derivative used in this synthesis, Jeffamine M-1000, was a commercial product with a nominal molecular mass of *ca* 1000. From ^{1}H NMR data we derived a structure containing an average of one propylene oxide unit randomly distributed in the molecular chain: $H_2NCH(CH_3)CH_2O[CH_2CH_2O]_{18}[CH(CH_3)CH_2O]_1CH_2CH_2OCH_3$. Poly-D,L-succinimide[42] (970 mg; 10 mmol), was dissolved in 25 ml of dry *N*,*N*-dimethylformamide. To the stirred solution was added a solution of dry Jeffamine M-1000 (5.0; g; 5 mmol) in the same solvent (10 ml). After saturation with N_2, the resulting solution was stirred for 24 h at 50 °C (bath temperature) with moisture protection, cooled in an ice bath, and resaturated with N_2, before a solution of diethylenetriamine (155 mg; 1.5 mmol) in 20 ml of *N*,*N*-dimethylformamide was added rapidly, by syringe, with strong agitation, again with protection from moisture. Stirring was continued for 10 h at 0–5 °C and another 16 h at 20–25 °C. After the addition of ethanolamine (732 mg; 12 mmol), stirring was continued once more for 6 h at ambient temperature, whereupon most of the solvent was removed under reduced pressure, and the polymeric product was precipitated with Et_2O (30 ml), washed with hot precipitant and redissolved in H_2O (30 ml). The aqueous solution was dialyzed for two days against H_2O in Spectra/Por 4 tubing, and the retentate was freeze-dried. The crude solid residue was thoroughly rewashed with boiling Et_2O for removal of traces of adsorbed Jeffamine and was redialyzed for 30 h against H_2O in Spectra/Por 6 wet tubing. Freeze-drying of the retentate and post-drying for two days at 70 °C in an Abderhalden tube gave 1.0 g (37.6%) of beige-colored, water-soluble solid; η_{inh}, 13 ml g^{-1}. ^{1}H NMR (D_2O, pH 10) δ: 4.7– 4.5, 7.8H (6.67H; CH asp); 4.1–3.45, 58H (53.7H; CH, CH_2, Jeff; CH_2OH); 3.4-3.2, 12.9H (13.85H; OCH_3; $CONHCH_2$); 2.9–2.5, 19.3H (19.3H; remaining CH_2); 1.2–1.0 ppm, 3.7H (3.2H; C-CH_3). Because of obvious variations in the degree of Jeffamine incorporation, expected in this polymer-homologous reaction type, it proved necessary in repeat experiments to

determine the composition of each reaction product individually by NMR spectroscopy as in the case described.

Conversion of the carrier to the ferrocenylated conjugates **6** followed the procedure used for the preparation of **3,** except that 2 equiv. of the active ester were used and the heating period at 65 °C was extended to 24 h. The water-soluble solid **6,** of a light tan–brown color, was collected in 65% yield; $_{inh}$, 10 ml g^{-1}. ^{1}H NMR (D_2O, pH 9) δ: 4.25–4.05, 7.2H (9H; cyclopentadienyl); 3.4–2.3 ppm, 37.2H (37.2H; OCH_3; CONH-CH_2; CH_2-CH_2-CH_2; CH_2-NH-CH_2; CH_2Asp; NH_2-CH_2). Analysis: calcd. for ($C_{95.17}H_{162.17}FeN_{19.16}O_{38.08}$) (2240.0) (**6**): C, 51.03; H, 7.30; Fe, 2.49; N, 11.97%; C/N, 4.97:1. Found: C, 49.87; H, 7.49; Fe, 2.25; N, 12.02%; C/N, 4.84:1.

The active ester derivative of the ferrocenylbutanoic acid used in these conjugation experiments, *N*-succinimidyl 4-ferrocenylbutanoate, was obtained by treatment of the free acid in ethyl acetate solution with 1.2 equiv. each of *N*-hydroxysuccinimide and *N*,*N*′-dicyclohexylcarbodiimide for 5 h at ice-bath temperature and for another 24 h at room temperature. Filtration from precipitated dicyclohexylurea by-product and washing of the latter with the same solvent were followed by solvent removal from the combined filtrate and washings. The residue was recrystallized from ethyl acetate. Small portions of the urea crystallizing in the first fractions were discarded. From the combined main fractions, recrystallized once more, the ester was collected as light orange crystals, m.p. 89–91 °C. Yield, 68%.

Cytoxicity testing *in vitro*

Cytotoxicity assays were performed as described by Van Rensburg *et al.*[43] using round-bottomed 96-well tissue culture plates. To each well were added 2500 tissue culture cells (HeLa human cervix epithelioid carcinoma; ATCC CC 42), and the volumes were brought to 200 µl with MEM supplemented with 10% fetal calf serum (FCS) containing the various drug concentrations or control systems. The plates were incubated for 72 h at 37 °C in 5% CO_2 and, at termination, fixed with 10% phosphate-buffered formalin, washed with phosphate-buffered saline and stained with 0.02% Crystal Violet. Plates were washed in water and the stain extracted with 10% sodium dodecyl sulfate. The absorbance was measured at 620 nm on a multiscan plate reader. Background values (medium only) were subtracted from each reading. Results of three separate experiments are either expressed as the mean percentage (± S.E.M.) cell growth of the respective (untreated) control systems (Figure 2) or are given as IC_{50} values, i.e. the mean drug concentration, in µg Fe ml^{-1}, required to reduce the absorbance to 50% of control and, thus, cause 50% cell growth inhibition (Table 1).

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