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Ferrocenes as potential chemotherapeutic drugs: Synthesis, cytotoxic activity, reactive oxygen species production and micronucleus assay

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Abstract

Three new ferrocene complexes were synthesized with 4-(1*H*-pyrrol-1-yl)phenol group appended to one of the Cp ring. These are: 1,1'-4-(1*H*-pyrrol-1-yl)phenyl ferrocenedicarboxylate, ("Fc-(CO₂-Ph-4-Py)₂"), 1,4-(1*H*-pyrrol-1-yl)phenyl, 1'-carboxyl ferrocenecarboxylate ("Fc-(CO₂-Ph-4-Py)CO₂H") and 4-(1*H*-pyrrol-1-yl)phenyl ferroceneacetylate ("Fc-CH₂CO₂-Ph-4-Py"). The new species were characterized by standard analytical methods. Cyclic voltammetry experiments showed that Fc-CH₂CO₂-Ph-4-Py has redox potential very similar to the Fc/Fc⁺ redox couple whereas Fc-(CO₂-Ph-4-Py)₂ and Fc-(CO₂-Ph-4-Py)CO₂H have redox potentials of over 400 mV higher than Fc/Fc⁺ redox couple. The *in vitro* studies on Fc-(CO₂-Ph-4-Py)₂ and Fc-(CO₂-Ph-4-Py)CO₂H revealed that these two compounds have moderate anti-proliferative activity on MCF-7 breast cancer cell line. In contrast Fc-CH₂CO₂-Ph-4-Py which displayed low anti-proliferative activity. In the HT-29 colon cancer cell line, the new species showed low anti-proliferative activity. Cytokinesis-block micronucleus assay (CBMN) was performed on these ferrocenes and it was determined they induce micronucleus formation on binucleated cells and moderate genotoxic effects on the MCF-7 breast cancer cell line. There is a correlation between the IC₅₀ values of the ferrocenes and the amount of micronucleus formation activity on binucleated cells and the reactive oxygen species (ROS) production on MCF-7 cell line.

Keywords

ferrocene; anti-proliferative; cytokinesis-block micronucleus assay; MCF-7; HT-29; MCF-10A

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1. Introduction

Drug resistance and toxic side effects are the major challenges encountered in chemotherapy treatments. Researchers have driven their interests into the development of metallic drugs, other than platinum compounds, to overcome some of the abovementioned problems. Among them, ferricenium and ferrocene derivatives opened a new ground in the discovery of new chemotherapeutic agents [1]. The versatility and ease of ferrocene functionalization has allowed the design and synthesis of a wide variety of ferrocene derivatives with biological activity [2–14].

Numerous pendant (functional) groups have been attached or linked to the Cp ring to tailor the anti-proliferative properties of ferrocene, many of them with great success [2–14]. Recently a new range of possible chemotherapeutic compounds have been studied using pyrrole derivatives. Those pyrrole derivatives have been demonstrated to have good anti-proliferative activity and an increase in membrane permeability allowing the compounds reaching the nucleus [15–22].

Our research group has been working for many years in the structure modification of the Cp ring with different pendant groups aimed to enhance the cytotoxic activity of the corresponding functionalized ferrocene [13,14]. One of our major accomplishment is the synthesis of ferrocenoyl 17 β -hydroxy-estra-1,3,5(10)-trien-3-olate complex (“Fc-CO₂-estradiol”) which exhibited enhanced anti-proliferative activity on hormone-dependent breast epithelial adenocarcinoma cell line MCF-7 [13]. Docking studies of this species into the alpha estrogen receptor (ER α) revealed that the ferrocene docks partially in the estrogen binding pocket impairing the protein function [13]. More recently, we published a series of ferrocenoyl esters which have anti-proliferative activities depending on the pendant group attached to the Cp ring [14]. Of particular interest is 4-bromophenyl ferrocenecarboxylate (Fc-CO₂-Ph-4-Br) which also displayed good anti-proliferative activity on breast cancer cell line MCF-7 and low anti-proliferative activity on non-tumorigenic human breast epithelial cell line MCF-10A [14]. Also a ferrocenecarboxylate modified with a pyrrole group, 4-(1H-pyrrol-1-yl)phenyl ferrocenecarboxylate, (Fc-CO₂-Ph-4-Py), has been reported showing good antiproliferative activity on MCF-7 [14]. In contrast to Fc-CO₂-estradiol, Fc-CO₂-Ph-4-Br anti-proliferative activity does not correspond to the anti-estrogenic properties and it is correlated to the cytotoxic effects of the ferrocene moiety.

In order to gain more insights regarding the structure-activity relationship of these metallocenes, we have prepared new ferrocene derivatives modified with pyrrole group and their anti-proliferative activities and mechanism of action have been studied and compared to Fc-CO₂-estradiol complex. Herein we report these new findings.

2. Experimental section

2.1 General procedure

All reactions were performed under an atmosphere of dry nitrogen using schlenk glassware or a glovebox, unless otherwise stated. Reaction vessels were flame dried under a stream of nitrogen, and anhydrous solvents were transferred by oven-dried syringes or cannula.

CH₂Cl₂ was dried and deoxygenated by distillation over CaH₂ under nitrogen. Infrared spectra were recorded on a Perkin Elmer Spectrum ATR spectrometer in solid. The NMR spectra were obtained on a 500MHz Bruker spectrometer. Electrochemical characterizations were carried out on a BAS CV-50W voltammetric analyzer. A Bruker Daltonics Esquire 6000 instrument was used to record the mass spectral data. The electrospray positive ion was used as ionization mode during the MS experiment. Elemental analyses were obtained from Atlantic Microlab Inc. Silica gel was heated at about 200°C while a slow stream of dry nitrogen was passed through it prior to use. 4-(1*H*-Pyrrol-1-yl)phenol, ferroceneacetic acid and ferrocenedicarboxylic acid were purchased from Aldrich and used as received.

Hydrolytic stability studies of Fc-(CO₂-Ph-4-Py)CO₂H, and Fc-CH₂CO₂-Ph-4-Py were performed by ¹H NMR using a solvent mixture of 5% DMSO-d₆ - 95% D₂O (v/v). The complex solutions were prepared first by dissolving the corresponding ferrocene in DMSO-d₆ and then D₂O was added to a final composition of 5% DMSO-d₆-95% D₂O. The ¹H NMR spectrum of the complex solution was then recorded at 0 h, 24 h and 72 h. Attempts to study the hydrolytic stability of Fc-(CO₂-Ph-4-Py)₂ was hampered by the limited solubility of this compound on aqueous solution.

2.2 Synthesis and characterization

2.2.1 Synthesis of 1,1'-4-(1*H*-pyrrol-1-yl)phenyl ferrocenedicarboxylate ("Fc-(CO₂-Ph-4-Py)₂")—In a 50 mL three neck round bottom flask, 1,1'-ferrocenedicarboxylic acid (0.2741 g, 0.001 mol), oxalyl chloride (174.5 μL, 0.002 mol) and pyridine (161 μL, 0.002 mol) were reacted in 20 mL of dichloromethane (DCM) under nitrogen atmosphere at room temperature. The reaction was carried out for 12 h to obtain 1,1'-ferrocenoyl dichloride. For esterification, 1,1'-ferrocenoyl chloride was reacted with 4-(1*H*-pyrrol-1-yl)phenol (0.1592 g, 0.001 mol) and pyridine (161 μL, 0.002 mol) for 12 h. After that period, three consecutive washing with 0.01 M HCl were carried out. The organic phase was then dried over sodium sulfate. From the reaction a mixture of three components were obtained; 4-(1*H*-pyrrol-1-yl)phenyl ferrocenecarboxylate, ("Fc-CO₂-Ph-4-Py") [14], 1,1'-4-(1*H*-pyrrol-1-yl)phenyl ferrocenedicarboxylate, ("Fc-(CO₂-Ph-4-Py)₂"), and 1-4-(1*H*-pyrrol-1-yl)phenyl, 1'-carboxyl ferrocenecarboxylate ("Fc-(CO₂-Ph-4-Py)CO₂H"). The products were separated by column chromatography using silica gel and DCM as mobile phase. The three products, Fc-CO₂-Ph-4-Py, Fc-(CO₂-Ph-4-Py)₂, and Fc-(CO₂-Ph-4-Py)CO₂H were isolated in 3–9% yield, 12–15 % yield, and 45–47 % yield respectively. Characterization of 4-(1*H*-pyrrol-1-yl)phenyl ferrocenecarboxylate, ("Fc-CO₂-Ph-4-Py") was previously reported [14].

1,1'-Ferrocenyl dichloride: ¹H NMR (500 MHz, CDCl₃) (δ ppm): 4.99 (4H, s), 4.72 (4H, s).

1,1'-4-(1*H*-pyrrol-1-yl)phenyl ferrocenedicarboxylate ("Fc-(CO₂-Ph-4-Py)₂"): ¹H NMR (500 MHz, CDCl₃) (δ ppm): 7.37 (2H, d, ph; ³J = 8.8 Hz), 7.25 (2H, d, py; ³J = 2.8 Hz), 7.03 (2H, dd, ph; ³J = 1.3 Hz), 6.34 (2H, dd, py; ³J = 1.6 Hz), 5.08 (2H, overlapping doublets, AA', Cp), 4.64 (2H, overlapping doublets, BB', Cp).

¹³C NMR (125 MHz, CDCl₃) (δ ppm): 169.0 (C=O), 148.3, 138.6, 122.9, 121.5, 119.5, 110.5, 73.4, 72.4, 72.0.

ATR-IR(cm^{-1}): 3145, 3092, 2925, 2841, 1727 (C=O), 1523, 1265, 1205, 1106, 848, 796, 727.

Anal. Calc. for $\text{C}_{32}\text{H}_{24}\text{O}_4\text{FeN}_2$: C, 69.05; H, 4.40 Found: C, 68.62; H, 4.46.

1-4-(1H-pyrrol-1-yl)phenyl, 1'-carboxyl ferrocenecarboxylate ("Fc-(CO₂-Ph-4-Py)CO₂H"): ^1H NMR (500 MHz, CDCl_3): 7.45 (2H, d, ph; $^3J = 8.5$ Hz), 7.31 (2H, d, py; $^3J = 8.5$ Hz), 7.08 (2H, dd, ph; $^3J = 1.6$ Hz), 6.37 (2H, dd, py; $^3J = 1.6$ Hz), 5.30 (CH_2Cl_2 , s), 5.01 (2H, overlapping doublets, AA', Cp), 4.95 (2H, overlapping doublets, BB', Cp), 4.57 (2H, overlapping doublets, AA', Cp'), 4.56 (2H, overlapping doublets, BB', Cp')

^{13}C NMR (125 MHz, CDCl_3) (δ ppm): 168.9 (C=O)', 166.4 (C=O), 148.3, 138.5, 122.9, 121.5, 119.5, 110.5, 74.3, 73.8, 73.4, 72.5, 72.1, 71.0, 53.5 (CH_2Cl_2).

ATR-IR (cm^{-1}): 3410(COOH), 3137, 3032, 2963, 1772(C=O), 1697 (C=O)', 1515, 1250, 1068, 1015, 825, 712.

ESI-MS (positive mode), m/z (relative intensity, 4): $[\text{Fc}-(\text{CO}_2\text{-Ph-4-Py})\text{CO}_2\text{H}]^+$ 413(5), 415(100), 416(23), 417(4). Anal. Calc. for $\text{C}_{22}\text{H}_{17}\text{O}_4\text{NFe}(\text{CH}_2\text{Cl}_2)_{0.125}$: C, 62.40; H, 4.08 Found: C, 62.59; H, 4.28.

2.2.2 Synthesis of 4-(1H-pyrrol-1-yl)phenyl ferroceneacetylate ("Fc-CH₂CO₂-Ph-4-Py")—In a 50 mL three neck round bottom flask, ferroceneacetic acid (0.0600g, 0.25 mmol), oxalyl chloride (21.8 μL , 0.25 mmol), pyridine (20.1 μL , 0.25 mmol) and 4-(1H-pyrrol-1-yl)phenol (0.0398g, 0.25 mmol) were reacted in 10 mL of dichloromethane (DCM) under nitrogen atmosphere at room temperature. The reaction was carried out for 12 h to get 4-(1H-pyrrol-1-yl)phenyl ferroceneacetylate. After that period three consecutive washing with 0.01 M HCl were carried out. The organic phase was then dried over sodium sulfate. The product was separated by column chromatography using silica gel and DCM as mobile phase. Fc-CH₂CO₂-Ph-4-Py was isolated in 62 % yield.

4-(1H-pyrrol-1-yl)phenyl ferroceneacetylate ("Fc-CH₂CO₂-Ph-4-Py"): ^1H NMR (500 MHz, CDCl_3): 7.44 (2H, d, ph; $^3J = 8.8$ Hz), 7.18 (2H, d, py; $^3J = 8.8$ Hz), 7.01 (2H, dd, ph; $^3J = 1.9$ Hz), 6.36 (2H, dd, py; $^3J = 1.9$ Hz), 5.30 (CH_2Cl_2 , s), 4.32 (2H, bs, Cp), 4.19 (2H, bs, Cp', 5H), 3.62 (2H, s, CH₂, 2H).

^{13}C NMR (125 MHz, CDCl_3) (δ ppm): 169.5 (C=O), 148.32, 138.6, 122.6, 121.6, 119.54, 110.5, 79.7, 68.8, 68.1, 53.5 (CH_2Cl_2), 35.6.

ATR-IR (cm^{-1}): 3092, 2963, 2917, 1750(C=O), 1508, 1334, 1143, 1098, 810, 727.

ESI-MS (positive mode), m/z (relative intensity, 4): $[\text{Fc-CH}_2\text{CO}_2\text{-Ph-4-Py}]^+$ 384(5), 386(100), 387(22). Anal. Calc. for $\text{C}_{22}\text{H}_{19}\text{O}_2\text{NFe}(\text{CH}_2\text{Cl}_2)_{0.06}$: C, 67.88; H, 4.94 Found: C, 67.99; H, 5.04.

2.3 Anti-proliferative studies

Biological activity was determined using the MTT assay originally described by Mossman [23,24] but using 10% Triton in isopropanol as a solvent for the MTT formazan crystals [23,24]. The breast adenocarcinoma cell line MCF-7 and MCF-10A were purchased from American Type Culture Collection (ATCC HTB22 and CRL-10317) and were grown under sterile conditions in a culture chamber at 37 °C and 95% Air/5% CO₂. Breast adenocarcinoma cell line MCF-7 was grown in Eagles's Minimum Essential medium supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) penicillin/streptomycin, and 0.01 mg/mL bovine insulin. Non-tumorigenic human breast epithelial cell line MCF-10A, was grown in Dulbecco's modified Eagle's medium (DMEM), 5% horse serum, 0.5 µg/mL hydrocortisone (Sigma Cat. no. H-0888), 10 µg/mL insulin (Sigma-ca. no. I1882), 20 ng/mL epidermal growth factor (Sigma Cat. no. E-9644). HT-29 cells were maintained at 37 °C and 95% Air–5% CO₂ in McCoy's 5A (ATCC) complete medium, which had been supplemented with 10% (v/v) fetal bovine serum (ATCC) and 1% (v/v) penicillin/streptomycin (Sigma). Asynchronously growing cells were seeded at 1.0×10^5 cells per well in 96-well plates containing 100 µL of complete growth medium, and allowed to recover overnight. Various concentrations of the complexes (10–1300 mM) dissolved in 5% DMSO–95% medium were added to the wells (eight wells per concentration; experiments performed in quadruplicate plates). The complex solutions were prepared first by dissolving the corresponding ferrocene in DMSO and then medium was added to a final composition of 5% DMSO–95% medium. In addition to the cells treated with the ferrocenes, two controls experiments were run: one without any addition of solvent mixture (5% DMSO–95% medium) and one adding 5% DMSO–95% medium to the cells. Both control experiments behaved identically, showing that 5% of DMSO in the medium did not render toxic to these types of cells. The cells were incubated for an additional 70 h. After this time, MTT dissolved in complete growth medium was added to each well to a final concentration of 1.0 mg mL^{-1} and incubated for two additional hours. After this period of time, all MTT containing medium was removed, cells were washed with cold PBS and dissolved with 200 µL of a 10% (v/v) Triton X-100 solution in isopropanol. After complete dissolution of the formazan crystals, absorbances were recorded in triplicate on a 340ATTC microplate reader (SLTLab Instruments) at 570 nm with background subtraction at 630 nm. Concentrations of compounds required to inhibit cell proliferation by 50% (IC₅₀) were calculated by fitting data to a four-parameter logistic plot by means of Sigma Plot software from SPSS (SPSS; Chicago, IL).

2.4 Electrochemistry

Cyclic voltammetric experiments were performed in deoxygenated CH₃CN solutions of ferrocene complexes with 0.1 M [NBu₄]⁺PF₆[−] as supporting electrolyte and ferrocene complex concentration of 2×10^{-3} M. The three electrodes used were platinum disk as the working electrode, Ag/AgCl as a reference electrode, and Pt wire as an auxiliary electrode. The working electrode was polished with 0.05 µm alumina slurry for 1–2 minutes, and then rinsed with double-distilled and deionized water. This cleaning process is done before each CV experiment and a sweep between 0 and 2000 mV is performed on the electrolyte solution to detect any possible deposition of ferrocene on the electrode surface.

2.5 Cytokinesis-block micronucleus assay (CBMN)

Cytokinesis-block micronucleus assay (CBMN) was performed as a modified procedure from Fenech M. [25]. Breast adenocarcinoma cells (MCF-7) were seeded in a 75 cm² flask with 20 mL of growth media and incubated for about 72 h. Following disaggregation with Trypsin/EDTA and re-suspension in complete medium, with concentration of 1×10^6 cells/mL, the cells were seed on a six well plate. After 24 h the medium was replaced with a solution at different concentrations of ferrocene complexes. Various concentrations (10 to 0.01 μ M) of the corresponding ferrocene were set below IC₅₀. Ferrocene solutions were prepared first by dissolving the corresponding ferrocene in DMSO and then medium was added to a final composition of 5% DMSO–95% medium. The different solutions of the desired ferrocene were added to individual wells, in addition to solutions of a negative control (cells without treatment) and a positive control (cell with methyl methane sulphonate, MMS). Methyl methane sulphonate concentration was selected according to Decordier *et al.* [26]. After 72 h of drug exposure, the solution of ferrocene complex was then replaced with fresh media containing only 6 μ g/mL of cytochalasin- β and incubated for another 24 h. The cells were washed with phosphate-buffer saline (PBS) and disaggregated with trypsin/EDTA. Following by centrifugation, the cells were re-suspended in 50 μ L of medium and then fixed with methanol and stained with hemacolor stain set (Harleco, cat# 65044-93). For each treatment condition, micronuclei in 500 binucleated MCF-7 cells for three experiments were scored (mean $\pm$ SD). Mitotic rate was assessed as per cent of binucleated cells (mean $\pm$ SD). Probability values (P) were estimated by Student's t-test comparing treated cells versus control.

2.6 Measurements of reactive oxygen species (ROS)

The ROS production was determined on MCF-7 treated with the oxidation-sensitive dye, 2',7'-dichlorodihydrofluoresceindiacetate (H₂DCF-DA, Sigma-Aldrich). The exposure of cells to H₂DCF-DA generates a fluorescent product 2',7'-dichlorofluorescein (DCF) which has a excitation/emission maxima of ~495/529 nm. The breast cancer cells (MCF-7) were seeded in 96-well plates at a concentration of 5×10^4 cells/mL containing 100 μ L of complete growth medium, and allowed to recover overnight. Various concentrations of the complexes (10–1300 mM) dissolved in 5% DMSO–95% medium were added to the wells (eight wells per concentration; experiments performed in quadruplicate plates). The complex solutions were prepared first by dissolving the corresponding ferrocene in DMSO and then medium was added to a final composition of 5% DMSO–95% medium. In addition to the cells treated with the ferrocenes, two controls experiments were run: one without any addition of solvent mixture (5% DMSO–95% medium) and one adding 5% DMSO–95% medium to the cells. Both control experiments behaved identically, showing that 5% of DMSO in the medium did not render toxic to these types of cells. The cells were incubated for an additional 70 h. After this time, the cells were treated with 10 μ M of H₂DCF-DA and incubated for 40 minutes at 37 °C after which time ROS production was evaluated at 605 nm.

3. Results and discussion

3.1 Synthesis and characterization

The subject ferrocenyl esters were synthesized by an established procedure reacting the 1,1'-ferrocenyl dichloride (Figure 1) and ferrocene acetyl chloride (Figure 2) with 4-(1H-pyrrol-1-yl)phenol. However, the first reaction (Figure 1) rendered not very efficient since in the synthesis of $\text{Fc}-(\text{CO}_2\text{-Ph-4-Py})_2$, the desired complex was isolated in low yield (12–15%) and it yielded two by-products: $\text{Fc-CO}_2\text{-Ph-4-Py}$ and $\text{Fc}-(\text{CO}_2\text{-Ph-4-Py})\text{CO}_2\text{H}$. Interestingly, $\text{Fc-CO}_2\text{-Ph-4-Py}$ was synthesized previously from ferrocenecarboxylic acid but in this synthesis it is the by-product of $\text{Fc}-(\text{CO}_2\text{-Ph-4-Py})\text{CO}_2\text{H}$ decarboxylation.

The structural characterization was very straightforward. The IR spectra showed bands in the $1727 - 1772\text{ cm}^{-1}$ region indicating the presence of the carbonyl stretching of the ester groups on the ferrocene. $\text{Fc}-(\text{CO}_2\text{-Ph-4-Py})\text{CO}_2\text{H}$ showed a second carbonyl stretching at 1697 cm^{-1} and a broad band at 3410 cm^{-1} indicating the presence of the carboxyl group attached on the other Cp ring. The ^{13}C NMR spectra exhibit signals about 169 ppm corresponding to the carbonyl groups. The $\text{Fc}-(\text{CO}_2\text{-Ph-4-Py})\text{CO}_2\text{H}$ showed a second carbonyl signal as expected at 166 ppm attributed to the carboxyl group. The ^1H NMR spectral data for $\text{Fc-CH}_2\text{CO}_2\text{-Ph-4-Py}$ showed a singlet corresponding to the unsubstituted Cp ring and two overlapping signals with AA'BB'' pattern corresponding to the monosubstituted Cp ring.

Likewise for $\text{Fc}-(\text{CO}_2\text{-Ph-4-Py})_2$ and $\text{Fc}-(\text{CO}_2\text{-Ph-4-Py})\text{CO}_2\text{H}$, the Cp signals appear as overlapping doublets in a AA'BB' pattern. The phenyl and pyrrole groups appear in the aromatic and conjugated vinylic regions respectively.

The hydrolytic stability of $\text{Fc-CH}_2\text{CO}_2\text{-Ph-4-Py}$ and $\text{Fc}-(\text{CO}_2\text{-Ph-4-Py})\text{CO}_2\text{H}$ was investigated by ^1H NMR in 5% DMSO d_6 –95% D $_2$ O solutions. $\text{Fc}-(\text{CO}_2\text{-Ph-4-Py})_2$ in this medium forms an emulsion and could not be investigated using this technique. At 24 h, $\text{Fc-CH}_2\text{CO}_2\text{-Ph-4-Py}$ and $\text{Fc}-(\text{CO}_2\text{-Ph-4-Py})\text{CO}_2\text{H}$ showed less than 5% of decomposition. At 72 h, $\text{Fc-CH}_2\text{CO}_2\text{-Ph-4-Py}$ was decomposed by 40% and $\text{Fc}-(\text{CO}_2\text{-Ph-4-Py})\text{CO}_2\text{H}$ by 30%. This is evidence that these ferrocenes are robust under pseudo-physiological conditions.

3.2 Electrochemical characterization

Electrochemical characterization of these new species was pursued by cyclic voltammetry since one of the proposed mechanism of cytotoxic activity involves the facile formation of ferrocenium ion in the cell [27,28]. As proposed by Osella et al., this redox property is responsible for the formation of reactive oxygen species (ROS) inducing oxidative damage to DNA [27,28]. Table 1 presents the redox properties of the subject complexes. The complexes have limited solubility in aqueous solution and soluble in 5% DMSO/90% H $_2$ O but when the $[\text{NBu}^n_4]\text{PF}_6$ is present the solution becomes cloudy. We selected CH $_3$ CN as solvent since it provided the best CV data.

In CH $_3$ CN solution, $\text{Fc}-(\text{CO}_2\text{-Ph-4-Py})_2$ ($E_{\text{pa}} = 958\text{ mV}$) and $\text{Fc}-(\text{CO}_2\text{-Ph-4-Py})\text{CO}_2\text{H}$ ($E_{\text{pa}} = 1027\text{ mV}$) showed oxidation potentials higher than Fc (494 mV), while $\text{Fc-CH}_2\text{CO}_2\text{-Ph-4-Py}$ showed similar oxidation potential ($E_{\text{pa}} = 503\text{ mV}$) to ferrocene. This data suggests that

the 4(1H-pyrrol-1-yl)phenylcarboxylate substituent is providing a place for electron delocalization away from the metal center and making them for robust to oxidation. For Fc-CH₂CO₂-Ph-4-Py, the CH₂ group between the ferrocene and the functional group avoid electron delocalization between the ferrocene unit and the 4(1H-pyrrol-1-yl)phenylcarboxylate. In light of the proposed mechanism of action and the possible correlation with the oxidation potentials, as a point of reference, it could be worth to compare these with previously reported potentials of biologically active ferrocene, Fc-CO₂-estradiol and 4-bromophenyl ferrocenecarboxylate (Fc-CO₂-Ph-4-Br), in MCF-7 and HT-29 cancer cell lines [13,14].

From the electrochemical point of view, Fc-CO₂-Ph-4-Br lacks of the pyrrole group and as a consequence, electron delocalization is reduced as well as the oxidation potential. Conversely, Fc-CO₂-estradiol has a steroid group which apparently is able to delocalize electrons and as a result higher oxidation potential. In spite of these differences, Fc-CO₂-estradiol and Fc-CO₂-Ph-4-Br have identical anti-proliferative activities in MCF-7 and HT-29 cancer cell lines thereby the correlation oxidation potential and cytotoxicity cannot be made. Attempts to study the redox behavior of these new species under physiological conditions were hampered by the limited solubility of these species in buffer or aqueous solutions. Nonetheless the current data provide an idea about the redox behavior of these species and can be used qualitatively.

3.3 Anti-proliferative activity studies

In vitro anti-proliferative activities of the new ferrocene complexes and their starting materials were determined by MTT-based assays involving a 72-h drug exposure on two malignant human cell lines, MCF-7 breast cancer and HT-29 colon cancer, and a normal healthy breast cell line, MCF-10A [23,24]. Their IC₅₀ values listed on Table 2 are compared with our two most active compounds at the present time in MCF-7 cell line, Fc-CO₂-estradiol and Fc-CO₂-Ph-4-Br [13,14]. In MCF-7 cell line, all derivatives showed dose dependent relationship with more anti-proliferative activity than their precursors (starting materials). Fc-(CO₂-Ph-4-Py)₂ (45.5(6) μM) and Fc-(CO₂-Ph-4-Py)CO₂H (57(7) μM) have moderate anti-proliferative activity on MCF-7 and low anti-proliferative activity non-tumorigenic human breast epithelial cell line MCF-10A. But their anti-proliferative activities on MCF-7 do not exceed those of Fc-CO₂-estradiol and Fc-CO₂-Ph-4-Br [13,14]. Fc-CH₂CO₂-Ph-4-Py has low antiproliferative activity on all cell lines, MCF-7, MCF-10A and HT-29. We should point out that none of the complexes have higher anti-proliferative activity on MCF-7 and HT-29 than Fc-CO₂-estradiol, and Fc-CO₂-Ph-4-Br.

3.4 Cytokinesis-block micronucleus assay

The genotoxic effects of Fc-CO₂-estradiol, Fc-(CO₂-Ph-4-Py)CO₂H, Fc-CH₂CO₂-Ph-4-Py, and Fc-(CO₂-Ph-4-Py)₂ were studied on MCF-7 cells using cytokinesis-block micronucleus (CBMN) assay after 72 h of treatment with these compounds. Micronuclei were scored in 500 binucleated MCF-7 cells as micronucleated binucleated cells. Figure 3 presents the photomicrographs of the MCF-7 cells in the CBMN assay without the presence of the drugs to demonstrate the appearance of mononucleated cell, binucleated cell and binucleated cell containing one micronucleus.

Three concentrations of each ferrocene complex were selected below IC_{50} values of the corresponding ferrocene. For Fc-CO₂-estradiol with an IC_{50} of 9(2) μ M the concentrations selected were 1 μ M, 0.1 μ M, and 0.01 μ M. For Fc-(CO₂-Ph-4-Py)₂, Fc-(CO₂-Ph-4-Py)CO₂H, and Fc-acPy with IC_{50} values between 45 to 103 μ M, the concentrations were 10 μ M, 1 μ M, and 0.1 μ M. The induction of micronucleus formation was characterized by an increase in single micronucleus in binucleated cells relative to a negative control that consists of untreated cells. Methyl methane sulphonate (MMS) was selected as positive control due to its well-known genotoxic activity [27]. Mononucleated, binucleated and micronucleated binucleated scored cells after 24 h of cytochalasin- β addition are represented Figure 3. Micronucleus formation in binucleated cells is associated to the genotoxic effect of the complex under study.

Figure 4 shows the micronuclei-forming activity of the ferrocene complexes as total micronucleated binucleated MCF-7 cells in 500 scored cells. Fc-CO₂-estradiol at concentrations of 1 μ M (mean 29, $P < 0.0001$) and 0.1 μ M (mean 25, $P < 0.01$) and Fc-(CO₂-Ph-4-Py)CO₂H at a concentration of 10 μ M (mean 25.5, $P < 0.01$) showed a 4-fold increase in micronucleus forming activity compared to the control experiment (untreated cells). At lower concentrations of both ferrocene complexes showed a decrease in micronucleus forming activity. At a concentration of 0.01 μ M (mean 24, $P < 0.0004$) Fc-CO₂-estradiol has 3-fold increase in micronucleus forming activity while Fc-(CO₂-Ph-4-Py)CO₂H shows similar micronuclei-forming activity for the two lower concentrations (1 μ M (mean 20, $P < 0.01$), and 0.1 μ M (mean 18.5, $P < 0.03$)). Fc-(CO₂-Ph-4-Py)₂ exhibits higher micronucleus forming activity than Fc-CO₂-estradiol and Fc-carbPy at a concentration of 10 μ M (up to 5-fold, mean 33.5, $P < 0.05$) meanwhile, at 0.1 μ M Fc-(CO₂-Ph-4-Py)₂ has no significant (mean 10, $P < 0.1$) increase in micronucleus forming activity compared to our control experiment. Fc-CH₂CO₂-Ph-4-Py has no significant ($P < 1$) micronucleus forming activity at any concentrations. Induction of single micronucleus formation in binucleated cells was found for Fc-CO₂-estradiol, Fc-(CO₂-Ph-4-Py)CO₂H, and Fc-(CO₂-Ph-4-Py)₂. Binucleated cells with more than one micronucleus were not significantly formed. Important differences in micronucleus forming activity depending on concentrations for Fc-CO₂-Estradiol, Fc-(CO₂-Ph-4-Py)CO₂H, and Fc-(CO₂-Ph-4-Py)₂ can be observed. For instance, a considerable increase in the number of total micronucleated binucleated cells at a concentration close to IC_{50} was observed for Fc-(CO₂-Ph-4-Py)₂. Likewise, Fc-CO₂-estradiol and Fc-(CO₂-Ph-4-Py)CO₂H showed similar genotoxic effect on MCF-7 cells at concentrations close to their IC_{50} . Fc-CH₂CO₂-Ph-4-Py with the lowest cytotoxicity appears to have no significant genotoxic effect on MCF-7 cells. For Fc-CO₂-estradiol, Fc-(CO₂-Ph-4-Py)CO₂H, and Fc-(CO₂-Ph-4-Py)₂, the micronucleus forming activity decrease as concentration decreases suggesting a dose dependent relation. The micronucleus forming activities in Fc-CO₂-estradiol, Fc-(CO₂-Ph-4-Py)CO₂H, Fc-CH₂CO₂-Ph-4-Py, and Fc-(CO₂-Ph-4-Py)₂ are lower than the activity for positive control (MMS) at all concentrations.

Mitotic rate effects on MCF-7 cells after 72 h treatment are presented on Table 3 as percent of binucleated cells following cytokinesis block. Statistical correlation of the percent of binucleated cells and untreated cells was estimated by Student's t-test. A decrease in the mitotic rate is observed upon a treatment of MCF-7 cells with all four ferrocene complexes

and the positive control (MMS), in comparison with untreated cells. However, those results reflect no significant differences ($P < 1$).

The micronucleus assay (CBMN) is a well-established technique used to measure the DNA damage on human or mammalian cells measuring the quantity of binucleated cells with micronuclei (MNi) [25,29]. Micronuclei (MNi) are whole chromosomes or chromosome fragments that result from DNA damage during anaphase nuclear division [29–34]. In the CBMN assay, once-divided, cells are the one that can express MNi and are recognized by their binucleated appearance [29–32].

Different mechanism of DNA damage may induce micronuclei-forming activity. Estrogens genotoxic mechanism may involve DNA adduct formation by estrogen metabolites, free radical by redox cycling of estrogens through microsomal, mitochondrial or nuclear processes [35–38].

For ferrocene derivatives, DNA damage may be caused by oxygen free radical species, inducing oxidative damage [12–14,27,28,39]. Those oxidative species cause DNA fragmentation resulting in micronucleated cells. In the present study four ferrocene derivatives were analyzed by CBMN assay to identify the amount of DNA damage and there is clear evidence that these species induce genotoxic effects.

The mechanistic route for Fc-CO₂-estradiol was recently elucidated. Fc-CO₂-estradiol binds to the alpha estrogen receptor, ER α , causing a conformational change acting as an antagonist [13]. Additionally, studies with confocal microscopy revealed that Fc-CO₂-estradiol migrate through the cell and reach the nucleus after 2 h of drug exposure [12]. Once Fc-CO₂-estradiol reaches the nucleus it generates oxygen free radical species resulting in DNA fragmentation.

The results for the micronucleus assay show an increase in micronucleus-forming activity up to 4-fold at a concentration near IC₅₀. These results are evidence of a moderate genotoxic effect of the Fc-CO₂-estradiol on MCF-7 cells. Meanwhile, studies reported by Yared *et al.* using β -estradiol on MCF-7 cells shows a 3-fold increase in the micronucleus-forming activity [35]. The differences in activity of Fc-CO₂-estradiol and β -estradiol can be attributed to the ferrocenoyl group.

The three ferrocene derivatives modified with 4-(1H-pyrrol-1-yl)phenol as pendant groups showed important differences in the anti-proliferative activity and genotoxicity. Fc-(CO₂-Ph-4-Py)CO₂H with a pyrrole group and a carboxylic group have an with IC₅₀ of 57 μ M while Fc-(CO₂-Ph-4-Py)₂ modified with 4-(1H-pyrrol-1-yl)phenol on each Cp ring have an with IC₅₀ of 45.5 μ M. Both Fc-(CO₂-Ph-4-Py)CO₂H and Fc-(CO₂-Ph-4-Py)₂ are the one with higher oxidation potentials than ferrocene. Fc-(CO₂-Ph-4-Py)CO₂H present up to 4-fold increase in micronucleus-forming activity close to IC₅₀ while Fc-(CO₂-Ph-4-Py)₂ shows up to 5-fold increase. The difference in micronucleus activity of these two ferrocene complexes may be attributed to the number of pyrrole groups attached to the Cp rings. Fc-(CO₂-Ph-4-Py)₂ contains two pyrrole groups while Fc-(CO₂-Ph-4-Py)CO₂H contains one. This pendant group, 4-(1H-pyrrol-1-yl)phenol, exhibit a oxidation potential close to 1.6 V providing an additional redox center which could induce redox processes (oxidative stress)

in the cell and enhancing the cytotoxic activity. In contrast, Fc-CH₂CO₂-Ph-4-Py which possess a CH₂ between the Cp ring and the pendant group and unsubstituted Cp ring exhibit the lower cytotoxicity, and lower genotoxic effect. As a result, it exhibits a mild reduction on mitotic rate compared to the untreated cells due to its low cytotoxicity.

A series of ferrocene derivatives using phenol pendant groups with various substituents on the 4-position (Fc-CO₂-Ph-4-X, X = F, Cl, Br, I, pyrrole) have been reported by our group [14]. *In vitro* studies on MCF-7 combined with docking studies on the ER α revealed that their anti-proliferative activities are not associated to the anti-estrogenic properties of the ferrocene but rather to the cytotoxic activity of the metallocene unit [14]. Noteworthy, the binding affinity of Fc-CO₂-Ph-4-Py in the ER α is lower than for the Fc-CO₂-Ph-4-X (X = F, Cl, Br, I) complexes [14]. Additionally, Fc-CO₂-Ph-4-Py has high anti-proliferative activity on hormone-dependent breast cancer cell line MCF-7 but it has also high anti-proliferative activity on non-tumorigenic human breast epithelial cell line MCF-10A. Thus, ER α is not the responsible for the anti-proliferative activity of Fc-CO₂-Ph-4-Py.

3.5 Measurements of reactive oxygenated species (ROS)

ROS induction assay was performed on MCF-7 to determine the relationship of the cytotoxic and genotoxic effect of the ferrocenoyl derivatives Fc-CO₂-estradiol, Fc-(CO₂-Ph-4-Py)CO₂H, Fc-(CO₂-Ph-4-Py)₂, and Fc-CH₂CO₂-Ph-4-Py, Figure 5.

Fc-CO₂-estradiol and Fc-(CO₂-Ph-4-Py)₂ which exhibit the higher micronucleus forming activity, also shows significant increase in ROS production. There is a direct correlation with ROS formation with concentration and a significant increase around 1000 μ M. The amount of ROS formation by Fc-CO₂-estradiol and Fc-(CO₂-Ph-4-Py)₂ are similar to FeCp₂ but lower than [FeCp₂]BF₄ as reported by C.Y. Acevedo et al. [39]. Although these results suggest that the genotoxic activities of Fc-CO₂-estradiol and Fc-(CO₂-Ph-4-Py)₂ are likely correlated to the ROS formation, other possible mechanisms cannot be ruled out. Along this line, the ROS formation of Fc-(CO₂-Ph-4-Py)CO₂H is the lowest but the IC₅₀ is comparable to Fc-(CO₂-Ph-4-Py)₂. Meanwhile, the ROS production for Fc-CH₂CO₂-Ph-4-Py, is low in comparison with FeCp₂ [39] and it has no relationship to concentrations. However, this last result was expected considering the low cytotoxic effect and micronucleus forming activity of Fc-CH₂CO₂-Ph-4-Py. This result suggests that genotoxic effect and ROS production of Fc-CH₂CO₂-Ph-4-Py is related to its poor cytotoxic activity.

4. Concluding remarks

In the present work, we have addressed the influence of the redox active pendant groups on ferrocene with regard to their anti-proliferative activity, genotoxic effects and ROS formation on breast cancer cell line. We utilized a combined approach using cancer cell lines and normal cell line to elucidate the real potential use of these species as chemotherapeutics and the cytokinesis-block micronucleus assay and ROS induction assay to measure the amount of DNA damage (genotoxic effects). We also performed cyclic voltammetry experiments to study the redox behavior of these new ferrocenes. First, in comparison to Fc-CO₂-estradiol and Fc-CO₂-Ph-4-Br which both have IC₅₀ values of 9.2 μ M but different redox potentials (756 and 532 mV respectively), we observe similar trends among the three

new ferrocene-pyrrole complexes. Given that the electrochemical studies were performed on CH₃CN, a non-physiological media, the redox data must be taken with caution and cannot explain the antiproliferative activities among these species. Second, Fc-CO₂-estradiol has the higher micronucleus-forming activity and on MCF-7 cell line followed by Fc-(CO₂-Ph-4-Py)₂ > Fc-(CO₂-Ph-4-Py)CO₂H > Fc-CH₂CO₂-Ph-4-Py. This trend is correlated to their IC₅₀ values. The ROS production assay confirmed the genotoxic activity of Fc-CO₂-estradiol and Fc-(CO₂-Ph-4-Py)₂ due to reactive oxygen species formation. Thus, this study provides evidences that the pendant groups have influence in the anti-proliferative activity and in less degree to the DNA damage on MCF-7 breast cancer cell line. The genotoxic effects of these new functionalized ferrocene complexes come mainly from the ferrocene moiety and not from the anti-estrogenic effects.

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Figure 1. Synthesis of ferrocenyl esters containing 4-(1H-pyrrol-1-yl)phenolate as pendant group. First and second complexes are the principal products of the reaction. The last product results from decarbonylation of the cyclopentadiene.

Figure 2.
Synthesis of 4(1H-pyrrol-1-yl)phenyl ferroceneacetate.

Figure 3.

Photomicrographs of the MCF-7 cells scored in the CBMN assay taken with an electronic microscope Olympus BX60. A) Mononucleated cell; B) binucleated cell; C) binucleated cell containing one micronucleus. All cells were treated with 6 μ g/mL Cytochalasin- β for 24 hours.

Figure 4.

Micronucleus forming activity of Fc-CO₂-estradiol, Fc-(CO₂-Ph-4-Py)CO₂H, Fc-CH₂CO₂-Ph-4-Py and Fc-(CO₂-Ph-4-Py)₂ on MCF7 cells (mean ± SD). Cells were seeded at a concentration of 1×10^6 cells per well on a six well plate, followed by 72 hours of drug exposure at different concentrations of the corresponding ferrocene. After treatment the cells were blocked at cytokinesis by addition of fresh medium containing 6 μg/mL cytochalasin-β. Cells were culture for a further 24 h prior to fixation and staining with methanol and Hemacolor Staining Set. Micronucleus formation was scored on 500 binucleated cells and compared to untreated cells as negative control and MMS as positive control.

Figure 5.

ROS production induced by (A)Fc-CO₂-estradiol, (B) Fc-(CO₂-Ph-4-Py)CO₂H, (C) Fc-(CO₂-Ph-4-Py)₂, and (D) Fc-CH₂CO₂-Ph-4-Py generated on MCF-7 at 72 h.

Table 1

Redox potentials of functionalized ferrocenes in CH₃CN with 0.1 M [NBu₄]ⁿPF₆ at a scan rate of 100 mVs⁻¹, using Ag/AgCl saturated as reference electrode.

Complexes	E _{pa} (mV)	E _{pc} (mV)	E (mV)	E _{1/2} (mV)
Fc-(CO ₂ -Ph-4-Py) ₂	958	862	96	910
Fc-(CO ₂ -Ph-4-Py)CO ₂ H	1027	901	126	964
Fc-CH ₂ CO ₂ -Ph-4-Py	503	431	73	467
* Fc-CO ₂ -estradiol	801	711	90	756
** Fc-CO ₂ -Ph-4-Br	580	484	96	532
Fc/Fc ⁺	494	414	80	454

* 13. Dalton Trans. 40 (2011) 9557–9565.

** 14. J. Organometal. Chem 749 (2014) 204–214.

Table 2

Anti-proliferative activities of ferrocene derivatives studied on MCF-7 breast cancer, MCF-10A normal breast, and HT-29 colon cancer cell lines, as determined using MTT assay after 72 h of drug exposure. IC₅₀ values based on quadruplicate experiments and standard deviation in parenthesis. N/A is non-active.

Complex	IC ₅₀ , MCF-7	IC ₅₀ , MCF-10A	IC ₅₀ , HT-29
Fc-(CO ₂ -Ph-4-Py) ₂	45.5(6)	298(5)	189(16)
Fc-(CO ₂ -Ph-4-Py)CO ₂ H	57(7)	219(21)	121(8)
Fc-CH ₂ CO ₂ -Ph-4-Py	103(6)	288(57)	283(11)
Fc-CO ₂ -estradiol	*9(2)	119(8)	*24.4(6)
Fc-CO ₂ -Ph-4-Br ¹⁴	**9.2(8)	**353(8)	24(6)
1-1'ferrocenedicarboxylic acid	N/A	N/A	410(21)
Ferroceneacetic acid	590(25)	86(7)	253(10)
4-(1H-pyrrol-1yl)phenol	249(5)	553(76)	211(44)
Ferricenium tetrafluoroborate ¹³	150		180

Table 3

Micronucleus forming activity values of control (untreated cells), MMS, Fc-CO₂-estradiol, Fc-(CO₂-Ph-4-Py)CO₂H, Fc-CH₂CO₂-Ph-4-Py, and Fc-(CO₂-Ph-4-Py)₂ on MCF-7 cells (mean ± SD) after 72 h treatment. Mitotic rate values represented as per cent binucleated MCF-7 cells (mean ± SD).

Ferrocene Complex	Concentrations (μM)	Micronucleus (MNI) (mean ± SD)	% Binucleated cells (Mitotic rate)
Control (untreated cells)		7 (4)	40 (15)
(MMS)	94.4	62 (6) ***	28 (3) *
Fc-CO ₂ -estradiol	1.0	29 (4) ****	34 (9) *
	0.1	25 (10) ***	39 (9) *
	0.01	23 (4) ****	33 (2) *
Fc-(CO ₂ -Ph-4-Py) ₂	10.0	34 (6) ***	25 (6) *
	1.0	15 (1) **	33 (13) *
	0.1	10 (1) **	18 (7) *
Fc-(CO ₂ -Ph-4-Py)CO ₂ H	10.0	26 (4) ***	12 (2) *
	1.0	20 (1) ***	16 (6) *
	0.1	19 (5) ***	13 (3) *
Fc-CH ₂ CO ₂ -Ph-4-Py	10.0	11 (7) *	38 (12) *
	1.0	8 (2) *	41 (15) *
	0.1	7 (4) *	33 (21) *

* P < 1,

** P < 0.1,

*** P < 0.05,

**** P < 0.0005 (treatment versus control) as determined by Student's t-test.