DOI: 10.1002/chem.200800507

Reactivity and Antiproliferative Activity of Ferrocenyl–Tamoxifen Adducts with Cyclodextrins against Hormone-Independent Breast-Cancer Cell Lines

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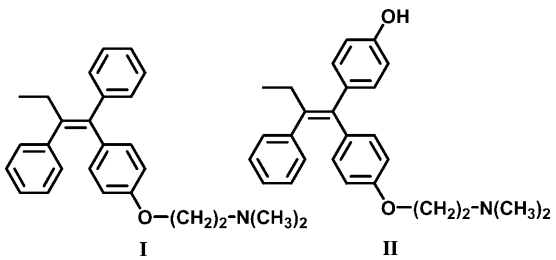
Abstract: Hydroxyferrocifen compounds are a new and promising class of ferrocifen-type breast-cancer drug candidates. They possess both endocrine-modulating properties and cytotoxic activity, which come from the tamoxifen skeleton and the presence of a ferrocene moiety, respectively. However, they suffer from reduced solubility in water, which presents a problem for their eventual therapeutic use. Herein, we examined the interactions of hydroxyferrocifen compounds with cyclodextrins (CDs) to evaluate whether or not their electron-transfer oxidation pathways were affected by their inclusion. It has been demonstrated that these inclusion complexes are soluble in pure water, which shows that CDs can be used to deliver these biologically active molecules. Therefore, a series of these compounds has been investigated by cyclic voltametry in various media in the presence of CDs (β-CD and Me-β-CD). In methanol, the hydroxyferrocifen compounds exhibited a weak interaction with the CD cavity. These interactions became stronger as the amount of added water increased. The complexation effect between the hydroxyferrocifen compounds and β-CD was found to be stronger if the CD was partially methylated, which is probably due to hydrophobic effects between the cyclopentadienyl ring and/or the aromatic rings and the methoxy groups. Moreover, it appears that the structure of the hydroxyferrocifen compounds affects both their solubility and their complexation dynamics. Investigations in the presence of pyridine show that the base kinetically favors the dissociation of the ferrocifen–CD complex during the electron transfer step, but does not affect the follow-up reactivity of the electrogenerated ferrocenium cation, which leads eventually to the corresponding quinone methide, as reported in the absence of CD. Accordingly, the cytotoxicity of these β-CD-encapsulated organometallic complexes in hormone-independent breast-cancer cells (MDA-MB231) were confirmed to be similar to those obtained in the absence of cyclodextrin.

Keywords: bioorganometallic chemistry · breast cancer · cyclodextrins · electrochemistry · iron

Introduction

Currently, the gold standard for endocrine therapy is tamoxifen (Novaldex; **I**), a selective estrogen receptor modulator (SERM) that inhibits cancer cell proliferation through its active metabolite hydroxytamoxifen (**II**) by competitively binding to the estrogen receptor (ER).[1–3]



I **II**

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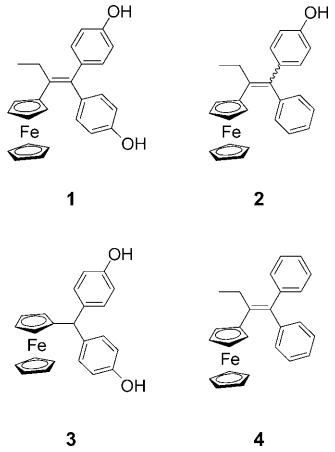
However, tamoxifen is active only against tumors that are estrogen-receptor positive (ER+), and frequently gives rise to resistance after prolonged use. As a consequence, the search for related but different agents has intensified considerably over the last few years.[4–8] Recently, it was discovered that grafting a ferrocenyl unit onto the tamoxifen skeleton allowed the preparation of a new and promising class of ferrocifen-type breast-cancer drug candidates.[9–12] These new molecules, called "hydroxyferrocifens" by analogy to tamoxifen, present the advantage of exhibiting dual functionality. Not only do they possess endocrine-modulating properties, but also cytotoxic activity. In the course of structure-reactivity relationship studies, various hydroxyferrocifens have been synthesized. To date, the most efficient compound in terms of pure cytotoxicity is the ferrocenyl diphenol compound **1** (Scheme 1). This compound exhibits a high antiproliferative activity in vitro against both hormone-dependent (MCF7, $IC_{50}=0.7\ \mu M$) and -independent (MDA-MB231, $IC_{50}=0.5\ \mu M$) breast-cancer cells.[13]



Scheme 1. Hydroxyferrocifen-type molecules studied herein in the presence of CDs.

Electrochemical studies of a series of hydroxyferrocifen compounds in methanol in the presence of a model base, pyridine, to establish the reactivity of these compounds in a model system have been reported.[14] Throughout this electrochemical survey, certain prerequisites were identified. These include the role of the ferrocene moiety as an intramolecular electron–hole reservoir, the conjugated π system as an electron-conjugating module, the presence of a phenol group as an electron donor, the requirement of a base, such as pyridine, to allow directional communication, and the constraints on the placement of the ethyl group. In summary, it was found that in the presence of a base, the oxidation of the ferrocene moiety promotes the oxidation of one phenol group into a quinone methide through a proton transfer coupled to an intramolecular electron-transfer mechanism. This is consistent with the previously observed chemical and biochemical oxidation of tamoxifen to its quinone methide form, which is expected to damage target cells by forming adducts with biological bases, such as DNA or proteins.[15–17] The high cytotoxicity of **1** was thus attributed to the lower oxidation potential of ferrocene and the ensuing stability of the ferrocenium moiety in comparison to phenol. The ferrocene moiety would act as an intramolecular oxidation "antenna", which thus allows the long-range displacement of the electron-transfer-activated molecule from the electron-accepting center to the target molecule, DNA, at which the quinone methide would form. This allowed the production of proquinoid species in milder oxidizing (i.e., physiological) conditions and simultaneously produced the effective nucleophilic agent only on specific targets.

Nevertheless, these promising hydroxyferrocifen compounds are not sufficiently soluble in water to allow their direct administration. This may limit both their bioavailability for future possible clinical applications and also the study of their activity under conditions that are similar to the cellular medium.

Various drug-delivery techniques, such as surfactant addition or salt selection, have been developed to overcome limitations that are linked to solubility and bioavailability, but the most popular procedure remains the inclusion of complexes within cyclodextrins (CDs).[18,19] CDs are cyclic oligomers of glucose that typically contain 6, 7, or 8 glucose monomers joined by α-1,4 linkages. These oligomers are commonly called α-CD, β-CD, and γ-CD, respectively. CDs form tori with hydrophobic interiors and hydrophilic exteriors. The amphiphilic character of CDs thus allows the solubilization in water of a wide variety of hydrophobic guest molecules by forming inclusion complexes.[20–23] The driving forces for the inclusion complexation of CDs with substrates are attributed to several factors, such as van der Waals forces, hydrophobic interactions, and electronic effects, and are ultimately controlled by steric factors owing to the specific structure of the torus interior.[24] Though CDs have been widely used for improving drug solubility and bioavailability, their chemical modification is often required to increase their own aqueous solubility. This is achieved by adding a small number of substituents to the hydroxyl groups of the glucose monomers. For instance, hydroxybutenyl-β-CD (HBenBCD) is very effective at solubilizing a broad spectrum of drugs, such as ibuprofen, glibenclamide,[25] tamoxifen,[26] raloxifen,[27] and itraconazole.[28] Alternatively, nanospheres and nanocapsules of amphiphilic β-cyclodextrin modified with aliphatic esters on the secondary face have been prepared to encapsulate tamoxifen citrate.[29] A new procedure that avoids the use of organic solvents and surfactants has been developed recently.[30] The formation of supramolecular nanoassemblies (nanogels) with a spherical shape has been achieved by the spontaneous association of two polymers, that is, a linear dextran and a cross-linked polycyclodextrin polymer. The inclusion of tamoxifen by the empty CD units could thus be achieved.

In this context, we took advantage of our experience with CDs to attempt to solubilize these active hydroxyferrocifens in aqueous media and to study their properties by using

electrochemistry.[31,32] The ability of CDs to include part of an organometallic complex in their internal hydrophobic cavities has been amply demonstrated.[33] However, inclusion generally modifies the chemical, photochemical, and electrochemical properties of organometallic compounds. To date, the organometallic compounds that have been most widely studied in the presence of CDs are ferrocenyl derivatives. In 1975, R. Breslow and B. Siegel were the first to report that ferrocene forms an adduct with β-CD in organic media.[34] A number of techniques, such as circular dichroism and cyclic voltammetry, have been used to establish the stoichiometry of the inclusion complexes and to investigate the nature of the host–guest interactions and association constants of the substrates with the CDs.[33] The stoichiometry of these complexes was found to depend on the size of the CDs. Thus, β-CD and γ-CD form 1:1 stoichiometric inclusion complexes, whereas α-CD forms a 2:1 complex with ferrocene. Moreover, it has been demonstrated that the orientation of ferrocene in β-CD is nearly parallel to the molecular axis of β-CD, whereas it is perpendicular to the molecular axis of γ-CD. Interestingly, calculations to establish the structure of the β-CD complex indicate that ferrocene may adopt both axial and equatorial orientations in the CD cavity so that the ultimate position is certainly imposed by the nature of the ferrocene substituents and by the external medium.

CD–ferrocene complexes have been used in various applications, such as the elaboration of mediators and molecular sensors, the assistance of organic reactions (e.g., asymmetric inductions), and condensation reactions.[33] In this work, CDs were used to solubilize various hydroxyferrocifen compounds that demonstrate biological activity against breast-cancer cell lines (Scheme 1). Cyclic voltammetry experiments were performed to investigate the inclusion of these substrates inside the CD cavity, and to determine the feasibility of generating the ferrocenium-centered cation in water and monitor its ensuing reactivity in the presence of both cyclodextrin and pyridine (used as a base). The in vitro effect of organometallic complexes **1** and **3** alone and encapsulated in β-CD were also investigated by using the hormone-independent breast-cancer cell lines MDA-MB2321. This work is of great importance for the examination of formulation strategies and completes a previous study that established that **1** could be incorporated in nanospheres and nanocapsules made from polyethyleneglycol polymer.[35]

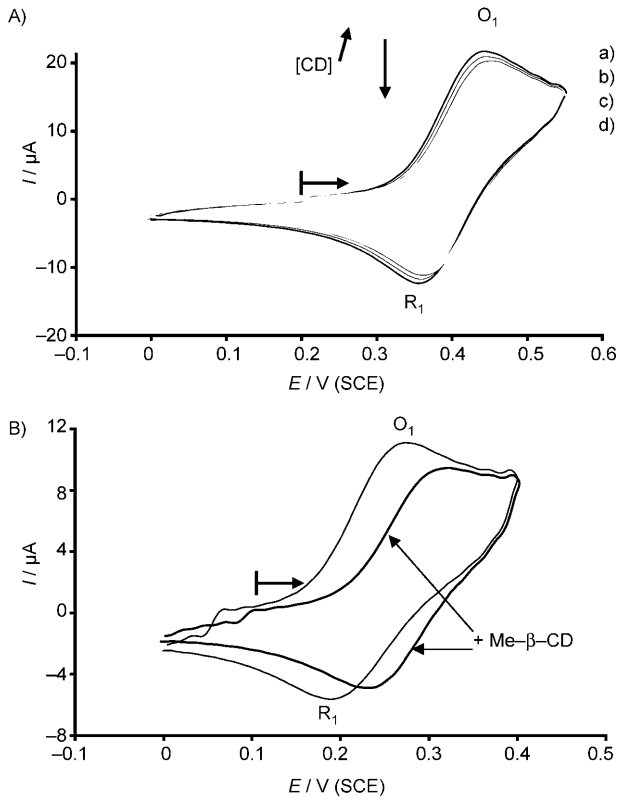


Figure 1. A) Cyclic voltammograms of **1** (1 mM) in MeOH with $TBABF_4$ (0.1 M) as the supporting electrolyte, recorded at a glassy carbon electrode (3 mm diameter) at a scan rate of 200 $mV\,s^{-1}$, a) in the absence of Me-β-CD or b,c,d) in the presence of 5, 10, and 15 equiv, respectively, of Me-β-CD. B) Cyclic voltammograms of **1** (1 mM) in $MeOH/H_2O$ (1:1) with $TBABF_4$ (0.1 M) as the supporting electrolyte, recorded at a glassy carbon electrode (3 mm diameter) at a scan rate of 200 $mV\,s^{-1}$, in the absence of and presence of Me-β-CD (5 equiv). Note the shift in the O_1/R_1 couple due to the presence of water (see Figure 1A).

Results and Discussion

Complexation of ferrocifen compounds with CDs in methanol and $MeOH/H_2O$: A typical cyclic voltamogramm of **1** in methanol at a glassy carbon electrode is displayed in Figure 1A. As already observed,[14] the reversible signal deals exclusively with the ferrocene/ferrocenium (Fc/Fc^+) redox couple, which shows that in the absence of a base, the possible extended delocalization plays no role.[14]

Adding increasing amounts of a randomly methylated β-CD (Me-β-CD) led to a slight decrease in the peak current of the O_1 wave, along with a shift in the peak potential towards more positive values. However, the peak retained its reversible shape. This effect became more dramatic as water was added to the methanolic solution, that is, when the solvent polarity was increased. A typical voltammogram obtained in $MeOH/H_2O$ (1:1 v/v) is shown in Figure 1B. These effects, which are similar to those obtained by D. H. Evans et al. for ferrocene carboxylic acid in the presence of β-CD, clearly demonstrate the effective inclusion of the apolar ferrocene moiety of **1** in the cavity of Me-β-CD.[36] The potential shift is indeed connected to the fact that **1** is more difficult to oxidize in the presence of CD because the neutral form is more strongly bound than its cationic oxidized form. On the other hand, the decrease in the peak current of O_1 is caused by the fact that the wave features the oxidation of the bulky and more slowly diffusing inclusion complex **1**·CD. As expected, the inclusion of **1** in the CD cavity is favored as the solvent polarity increases. The magnitude of these effects also depends on the nature of the CD because this affects complexation and diffusivity.

Figure 2 compares the cyclic voltammograms obtained for the same concentration of **1** in MeOH/H_2O (1:1) in the absence of and presence of 10 equivalents of either Me-β-CD

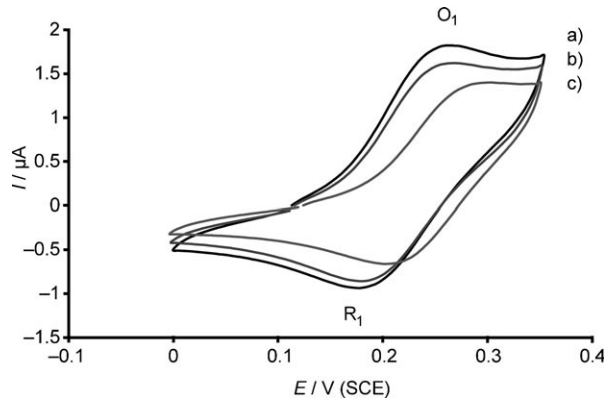


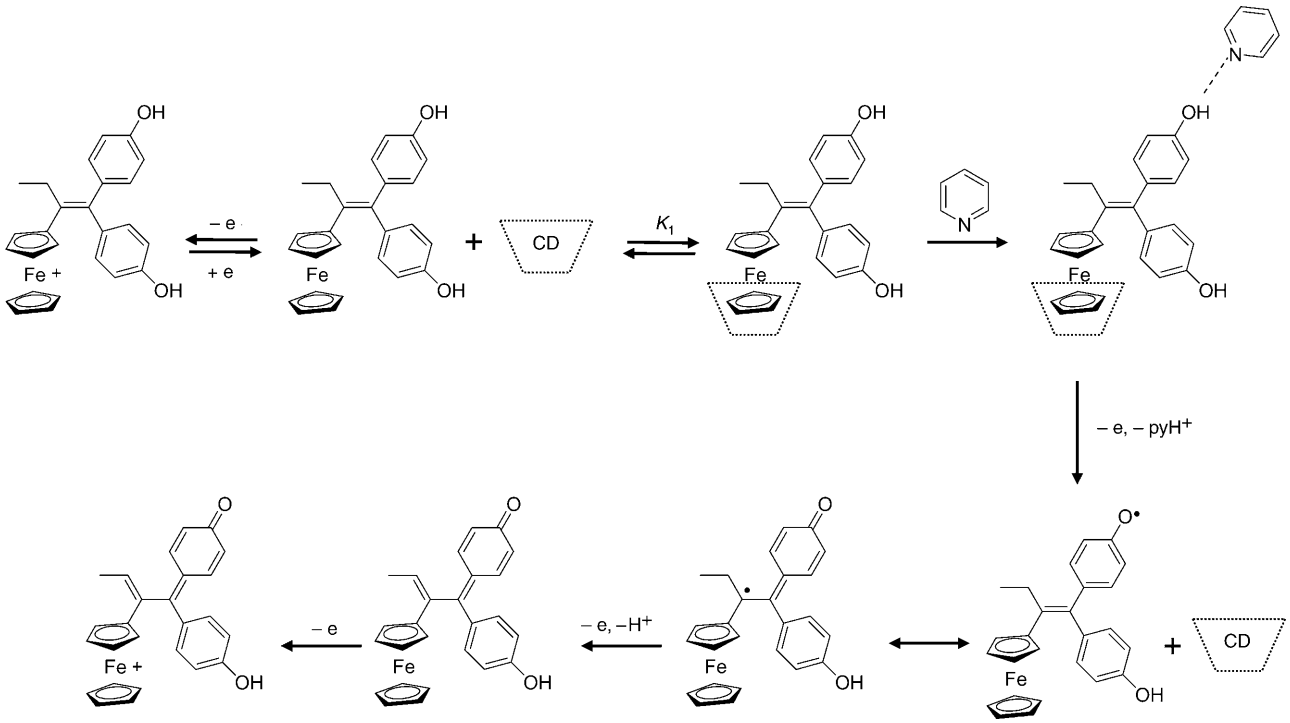
Figure 2. Cyclic voltammograms of **1** (1 mM) in MeOH/H_2O (1:1) with $TBABF_4$ (0.1 M) as the supporting electrolyte, recorded at a glassy carbon electrode (3 mm diameter) at a scan rate of 50 $mV s^{-1}$, a) in the absence of CD or b,c) in the presence of 10 equiv of β-CD and Me-β-CD, respectively.

or native β-CD. Figure 2 clearly shows that both the decrease in I_{O_1} and the shift in E_{O_1} are greater in the presence of Me-β-CD than β-CD, which demonstrates a stronger complexation effect in the former case. This certainly indicates that the strongest hydrophobic interactions are between the cyclopentadienyl and/or the aromatic rings and the methoxy groups of the partially methylated CD. Another interesting feature is the trend of the O_1 wave to achieve a sigmoidal shape, which is characteristic of the progressive involvement of a consecutive chemical–electrochemical (CE) mechanism (see Scheme 2). Indeed, under pure kinetic control, the current on the forward scan exhibits a plateau rather than a peak.[37] Such behavior has also been observed by D.H. Evans et al.[36] This confirms that when the electroactive guest forms a stable inclusion complex with a CD host, the complex generally cannot undergo any direct electrochemical reaction.[36–38] To allow the electron-transfer activation to take place, the complex must dissociate and release the electroactive moiety, which leads to a CE process. This has been fully substantiated for ferrocene derivatives, reduced viologens, and cobaltocene in particular.[33]

The thermodynamic stability of the **1**·Me-β-CD complex was evaluated in MeOH and MeOH/H_2O (1:1) by plotting the variation of the half-wave potential of O_1 ($\Delta E_{1/2}$) in the presence of CD ($(E_{1/2})_{app}$) with respect to the half-wave potential in the absence of CD ($E_{1/2}$) as a function of the total concentration of Me-β-CD (Figure 3). In both solvents, $(E_{1/2})_{app}$ shifted to more positive potentials as the CD concentration, [CD], was increased. As already reported, the relationship between $(E_{1/2})_{app}$ and [CD] can be depicted as follows:[39–41]

$$\Delta E_{1/2} = \frac{RT}{2F} \ln \left(\frac{(1+K_0[CD])(1+K_0 r[CD])}{(1+K_+[CD])(1+K_+ r[CD])} \right) \quad (1)$$

In Equation (1), K_0 and K_+ are the complexation constants between CD and **1** or $\mathbf{1}^+$, respectively, r is defined as $r = D_c/D_f$, in which D_f and D_c are the diffusion coefficients



Scheme 2. Electrooxidation of **1** in the presence of CD in the absence and the presence of pyridine.

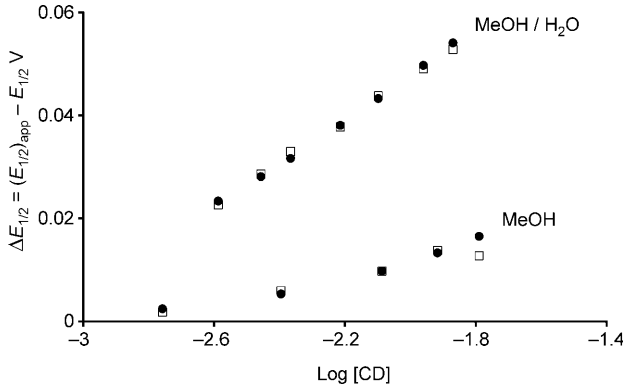


Figure 3. Half-wave potential difference of **1** in MeOH or MeOH/H_2O (1:1) in the presence of Me-β-CD ($(E_{1/2})_{CD}$) and in the absence of Me-β-CD ($E_{1/2}$) as a function of the total concentration of Me-β-CD, recorded at a glassy carbon electrode at a scan rate of 100 mV s^{-1}. □: Experimental values and ●: calculated values from Equation (1).

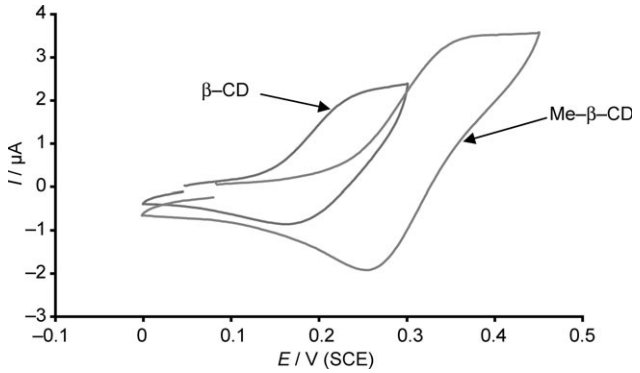


Figure 4. Cyclic voltammograms of **1** (1 mM) in H_2O with Na_2SO_4 (0.1 M) as the supporting electrolyte, recorded at a glassy carbon electrode (3 mm diameter) at a scan rate of 50 mV s^{-1} and in the presence of β-CD or Me-β-CD (20 equiv).

of **1** (free species) and the **1**·CD complex. The value of *r*, 0.49, was estimated by using the equation $r=(M_{\mathbf{1}}/M_{\mathbf{1}\cdot CD})^{1/2}$, in which $M_{\mathbf{1}}$ and $M_{\mathbf{1}\cdot CD}$ are the molecular weights of **1** and **1**·CD complex, respectively.[42]

A good correlation was obtained between the experimental and calculated values for $K_0=825\,M^{-1}$ and $K_+\leq 5\,M^{-1}$ in MeOH/H_2O. Similarly, in MeOH the best agreement with the experimental results was obtained for $K_0=80\,M^{-1}$ and $K_+\leq 1\,M^{-1}$, which confirmed a stronger complexation effect between **1** and Me-β-CD as the polarity of the medium increased. This also confirms that the positively charged ferrocinium compound is poorly complexed to the lipophilic cavity of the CD.

Complexation of ferrocifens with CDs in water: These results prompted us to investigate the effectiveness of solubilizing **1** in water by the addition of CDs. In the absence of CD, **1** was, as expected, totally insoluble and remained on the surface of the solution, and no electrochemical wave could be observed. In the presence of a large excess of Me-β-CD, the colorless solution turned yellow after few hours and the substrate was totally solubilized after one night. Note that because the CE kinetics, as observed above, are reasonably fast, this long delay is due to the extremely low concentrations of **1** in the water containing the CD molecules. In other words, the solubilization would be faster in the presence of a higher concentration of **1**. Typical cyclic voltammograms obtained under these conditions after complete solubilization are shown in Figure 4.

As expected, the oxidation of **1** in water occurred at a more positive potential than the studies performed in MeOH and MeOH/H_2O, which is in agreement with a stronger complexation effect between **1** and the CD cavity. The oxidation wave exhibits a more pronounced plateau shape, which thus features a fully developed CE process that proceeds under pure kinetic control. On the contrary, the current on the reverse scan exhibited a peak rather than a plateau, which confirms that the electrogenerated ferrocenium species was much less complexed (if indeed any complexation occurred) by the CD than the ferrocene derivative. Interestingly, when the same experiment was performed with β-CD instead of Me-β-CD, the oxidation peak, O_1, appeared at a less positive potential value and its intensity was smaller, which demonstrated that **1** is more strongly complexed by the randomly methylated β-CD (Figure 4). In agreement with this rationale, the peak current of O_1 was smaller in the presence of β-CD because a smaller amount of **1** could be solubilized in this case. On the other hand, the cyclic voltammogram obtained for authentic ferrocene ([$FeCp_2$], Cp = cyclopentadienyl) in the presence of β-CD and under conditions identical to those in Figure 4 gave no significant electrochemical response (the [$FeCp_2$]·CD complex was not soluble in water). This suggests that in the case of ferrocifens, the presence of the phenol groups, which presumably extend from the cavity, favor the higher solubility of the inclusion complex. In line with this view, the replacement of β-CD by Me-β-CD made the [$FeCp_2$]·Me-β-CD complex sufficiently soluble in water to produce an observable electrochemical signal, in agreement with the higher solubility of Me-β-CD compared with β-CD.[23] Because the strongest complexation effect was obtained in water and in the presence of Me-β-CD, other hydroxyferrocifen compounds (Scheme 1) that also possess a significant biological activity have been investigated under these optimized conditions (Figure 5).

Qualitatively, the time required to dissolve complexes **1**–**4**, either partially or totally, decreased in the order **4** > **2** > **1** > **3**. Here again, a comparison of the cyclic voltammograms of **4**, **2**, and **1** showed that the presence of phenol groups was of great importance for solubilizing the ferrocifen-CD adducts. Indeed, compounds **1** and **2** were totally solubilized after one day, whereas **4** was only partially dissolved after two days. This also explains the much lower oxidation current of **4** compared with those of **2** and **1**. Additionally, the peak current of O_1 was smaller for **1** than for **2**. This is probably due to a stronger complexation effect for **1**, which is in agreement with a stronger trend to achieve a pla-

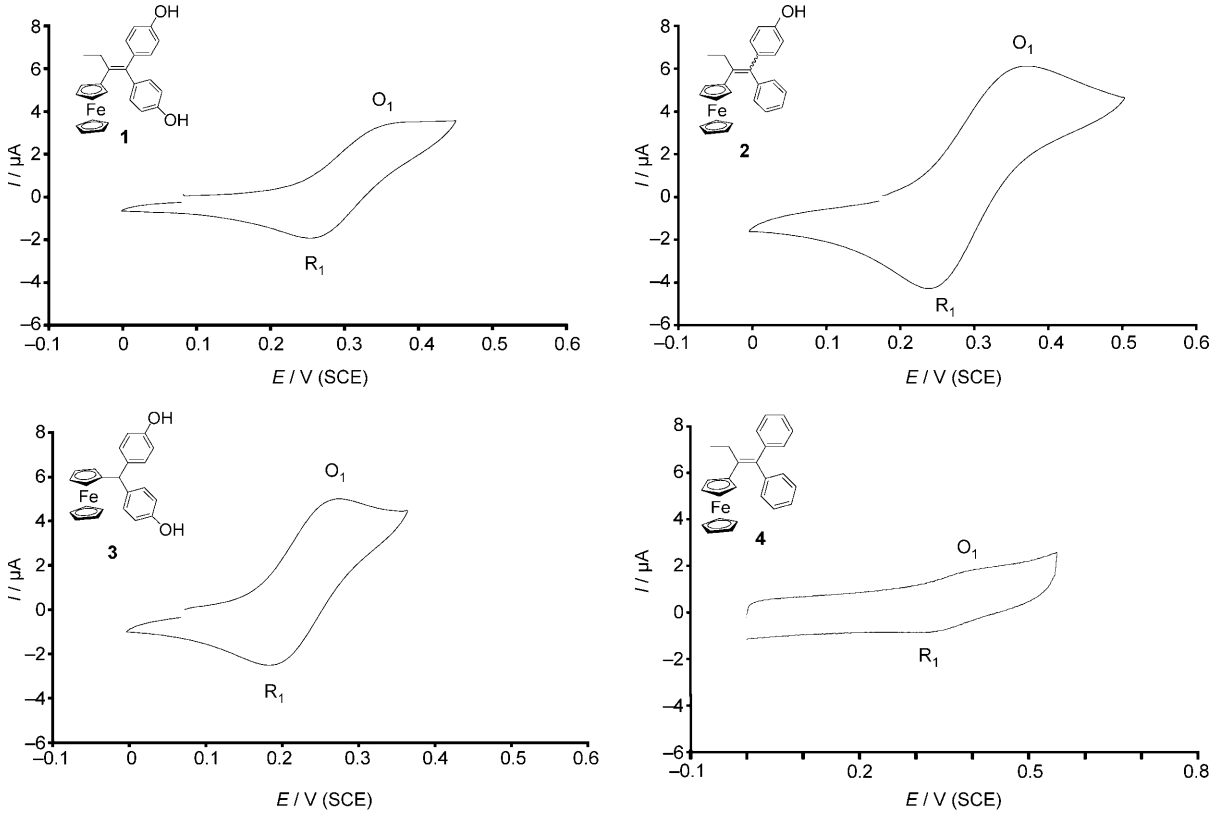


Figure 5. Cyclic voltammograms of hydroxyferrocifen-type molecules **1**–**4** (1 mM) in H_2O with Na_2SO_4 (0.1 M) as the supporting electrolyte, recorded at a glassy carbon electrode (3 mm diameter) at a scan rate of 50 $mV\,s^{-1}$ in the presence of Me-β-CD (20 equiv).

teau shape for **1**, because this indicates that decomplexation prior to electron transfer (CE mechanism) is slower for **1** than for **2**. As expected, the absence of the apolar ethylvinyl fragment increased the solubility of the complex (Figure 5, **1** and **3**). However, according to the cyclic voltammogram shape, the complexation dynamics were faster for **3** than for **1**.

Fate of electrogenerated ferrocenium cations from CD adducts and biological activity of β-CD-encapsulated complexes 1 and 3 on MDA-MB231 cells: Recently, cyclic voltammetry has been used to rationalize the biological reactivity of ferrocifen compounds in a model environment.[14] Indeed, breast-cancer cell death observed in the presence of the family of ferrocene derivatives shown in Scheme 1 has been ascribed to the in situ oxidation of phenol groups through intramolecular electron transfer to the ferrocenium cation.[43] The cyclic voltammograms obtained in MeOH in the presence of pyridine provided evidence that an intramolecular electron transfer from the phenol moiety to the ferrocenyl group occurred after the first ferrocene-centered electrooxidation, that is, when deprotonation of the transient phenol cation radical occurs in the presence of a suitable base. This led to a phenoxyl radical species that could be easily oxidized further into a reactive quinone methide that, in our view, could be responsible for cell death by the facile formation of adducts with DNA, GSH (GSH = glutathione), or proteins.[15–17] Note also that due to the mesomeric conjugation of the radical, this one-electron activated species may also give rise to a quinone methide species after a hydrogen atom transfer (note that $-e^-$, $-H^+$ is equivalent to "$-H^{\bullet}$"). Therefore, it is not clear that a two-electron activation is required under biological conditions, though this is spontaneous under electrochemical conditions.

In this context it was of great interest to us to investigate the reactivity of oxidized ferrocene derivatives in water in the presence of CDs, to examine whether or not the presence of CD affected the reactivity of the initially formed ferricinium cations. As shown in Figure 6, the addition of an excess of pyridine led to an approximately threefold increase of the peak current of O_1 and a pronounced change in the wave shape, which indicates a faster electron transfer and a more complex mechanism. Considering the initial sluggish one-electron CE process current, it may be inferred that the maximum current value obtained in the presence of pyridine roughly corresponds to a three-electron process. This electrochemical behavior shows that the presence of pyridine kinetically favors the dissociation of the **1**·CD complex and opens a subsequent two-electron pathway after the initial formation of the ferricinium derivative. This appears to be fully consistent with our former investigation of pyridine-induced effects on the voltammetry of **1** in apolar media.[14] This strongly suggests that the overall mechanism previously depicted for the oxidation of **1** in the presence of

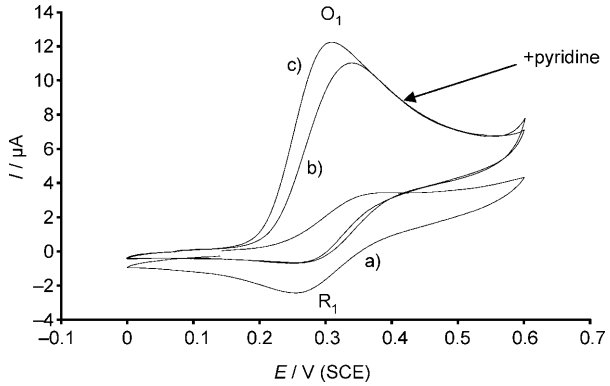


Figure 6. Cyclic voltammograms of **1** (1 mM) in H_2O with Na_2SO_4 (0.1 M) as the supporting electrolyte, recorded at a glassy carbon electrode (3 mm diameter) at a scan rate of 50 $mV\,s^{-1}$, in the presence of Me-β-CD (20 equiv) and a) in the absence of or b), c) in the presence of 0.1 and 0.2 M, respectively, of pyridine.

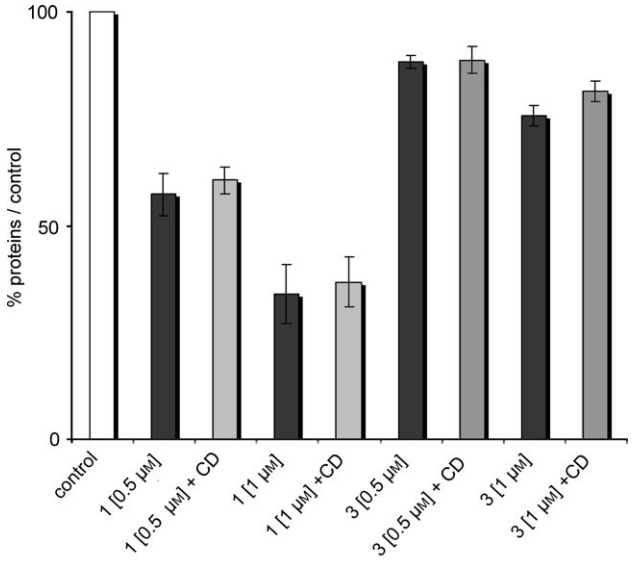


Figure 7. Comparison of the antiproliferative effect of ferrocenyl complexes **1** and **3** (0.5 or 1 µM) alone or encapsulated in Me-β-cyclodextrin (20 µM) on hormone-independent breast-cancer cell lines (MDA-MB231) after culturing for 96 h. Results are the average value of two independent experiments.

a base[14] still prevails when **1** is initially complexed by CD. Note that this is in complete agreement with the fact that oxidation of **1**·CD produces uncomplexed $\mathbf{1}^{\bullet+}$. So, a three-electron oxidation sequence is most likely triggered in the presence of pyridine, irrespective of the fact that **1** is initially included in a CD. The corresponding mechanism is summarized in Scheme 2. Note that the third electron transfer most likely corresponds to a further oxidation step for the ferrocene moiety of the quinone methide structure. Finally, the passage from a one-electron plateau shape to a three-electron peak shape for the O_1 wave in the presence of both CD and pyridine could result from the fact that pyridine interacts with the phenolic group of the **1**·CD adduct, which would allow the faster decomplexation of **1**. If pyridine reacted only with uncomplexed **1**, a plateau shape would still be observed with about a threefold current intensity. Accordingly, neutral species arising from the oxidation sequence may also exist as inclusion complexes. One may also consider a competitive complexation between pyridine and the ferrocene moiety with the CD host, which thus leads to a decrease in the association constant.

The in vitro effect of organometallic complexes **1** and **3**, alone or encapsulated in Me-β-CD, was investigated by adding them to hormone-independent breast-cancer cell lines (MDA-MB231) and culturing the mixtures for four days to assess whether or not the inclusion of **1** or **3** into CD affected their bioavailability (see the Experimental Section). The ensuing results are gathered in Figure 7. Clearly, no significant difference could be observed, the antiproliferative activities being identical, within the precision of the measurements, between the free and Me-β-CD-encapsulated forms. Compound **1** (1 µM) was still cytotoxic, as previously observed,[13] whereas **3** (1 µM) was only slightly cytotoxic.[44] A similar result was also observed with tamoxifen citrate β-cyclodextrin nanoparticles.[29] With respect to the association constant determined in $MeOH/H_2O$, one may also consider that the complexes are most likely dissociated at such low concentrations, although the polarity (and therefore the association constant) is much higher in pure water. Nevertheless, because **1** and **3** may be delivered at much more significant doses thanks to the higher solubilities of their inclusion complexes compared with the uncomplexed compounds, this opens encouraging routes for the production of more effective formulations of ferrocifens for anticarcinogenic therapies.

Conclusion

This work was devoted to the investigation of the behavior of hydroxyferrocifens compounds in the presence of CD vectors to examine a possible way to overcome their water insolubility and increase the bioavailability of these potent species, and to pave the way to defining better formulations of this new class of breast-cancer-active drugs. The results presented herein have established that the presence of hydroxyl groups on the phenol ring is essential to obtain ferrocifen–CD inclusion complexes that are soluble in polar solvents, which is in keeping with the fact that such derivatives are the most potent in antiproliferative investigations. Cyclic voltammetry allowed us to quantify and evidence the strong complexation effect that occurs between these organometallic compounds and CDs. Importantly, the complexation effect is more efficient with partially methylated β-CD than with native β-CD, which is most likely due to additional hydrophobic interactions. It was also shown that the presence of a base promotes the dissociation of the ferrocifen–CD complex upon electron transfer and that the reactivity of the electrochemically generated ferricinium cation in the pres-

ence of the base is not affected by the presence of the CD. More importantly, in the presence of β-CD the antiproliferative effect of **1** and **3** against hormone-independent breast-cancer cells (MDA-MB231) in water was similar to that obtained in the absence of cyclodextrin, thus opening the way for using this formulation in in vivo studies.

Experimental Section

Chemicals: Tetrabutylammonium tetrafluoroborate ($TBABF_4$) was used as the supporting electrolyte in studies in MeOH and MeOH/H_2O and was prepared from $NaBF_4$ (Acros) and nBu_4NHSO_4 (Acros), recrystallized from ethyl acetate/hexane (Acros), and dried at 60 °C. Na_2SO_4 (Aldrich) was used as the supporting electrolyte in studies in water. The water used was highly purified (resistivity = 18 MΩ cm; Milli-Q system; Millipore, Billerica, MA, USA). Methanol (Acros), DMSO (Acros), methyl-β-cyclodextrin, and β-cyclodextrin (Aldrich) were used as received. The ferrocifen complexes were synthesized by McMurray cross-coupling of the appropriate ketones. Their preparation is fully described elsewhere.[14] The solubilization of ferrocifen compounds in water was obtained after addition of cyclodextrin (20 M equiv). The mixture was stirred overnight before electrochemical runs were performed.

Instrumentation: Cyclic voltammetry experiments were performed at RT under an argon atmosphere in a three-electrode cell by using an Autolab potentiostat (PGSTAT 20). The reference electrode was an SCE (saturated calomel electrode; Tacussel), which was separated from the solution by a bridge compartment filled with the same solvent/supporting electrolyte solution as used in the cell. The counter electrode was a 1 cm gold wire. The glassy carbon working electrodes (3 mm diameter) were purchased from Radiometer Analytical.

Biological tests

Preparation of the solutions: For experiments in the absence of CD, 1×10^{-3} M stock solutions of the ferrocenyl complexes were prepared in DMSO. For inclusion experiments, a 2×10^{-3} M stock solution of Me-β-CD in water was prepared, then the ferrocenyl complexes were added and allowed to dissolve overnight. For biological activity assessments of the ferrocenyl complexes alone, a 1×10^{-3} M solution of **3** and a 5×10^{-4} M solution of **1** were prepared in DMSO and added to the culture medium to give the desired final dilutions (1×10^{-6} or 5×10^{-7} M). Note that the solubility of **3** was higher than that of **1**.

Culture conditions: Cells were maintained in a monolayer in DMEM (Dulbecco's modified Eagle medium) with phenol red (Gibco BRL), supplemented with fetal calf serum (8–9%; Gibco BRL) and glutamine (2 mM; Sigma) at 37 °C in a 5% CO_2/air-humidified incubator. For proliferation assays, cells were plated in 24-well sterile plates at a density of 1.1×10^4 cells (MDA-MB231) in DMEM (1 mL) without phenol red, supplemented with decomplemented and hormone-depleted fetal calf serum (10%) and glutamine (2 mM) and incubated. The following day (0 d), 1 mL of the same medium containing the compounds to be tested was added to the plates (final volume of DMSO: 0.1%; 4 wells for each condition). After 3 d, the incubation medium was removed and fresh medium that contained the compounds was added. After 4 d, the total protein content of the plate was analyzed by methylene blue staining; cell monolayers were fixed for 1 h in methanol, stained for 1 h with methylene blue (1 mg mL^{-1}) in phosphate-buffered saline, then washed thoroughly with water. HCl (1 mL, 0.1 M) was then added and the absorbance of each well was measured at $\lambda = 620$ nm with a Biorad spectrophotometer. The results presented here are expressed as a percentage of the proteins versus the control.

Acknowledgement

We thank the Agence Nationale de la Recherche for financial support (grant number: ANR-06-BLAN-0384-01, "FerVect").

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Received: March 18, 2008
Published online: July 30, 2008

