Multifaceted chemical behaviour of metallocene (M = Fe, Os) quinone methides. Their contribution to biology

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Multifaceted Chemical Behaviour of Metallocene (M = Fe, Os) Quinone Methides. Their Contribution to Biology

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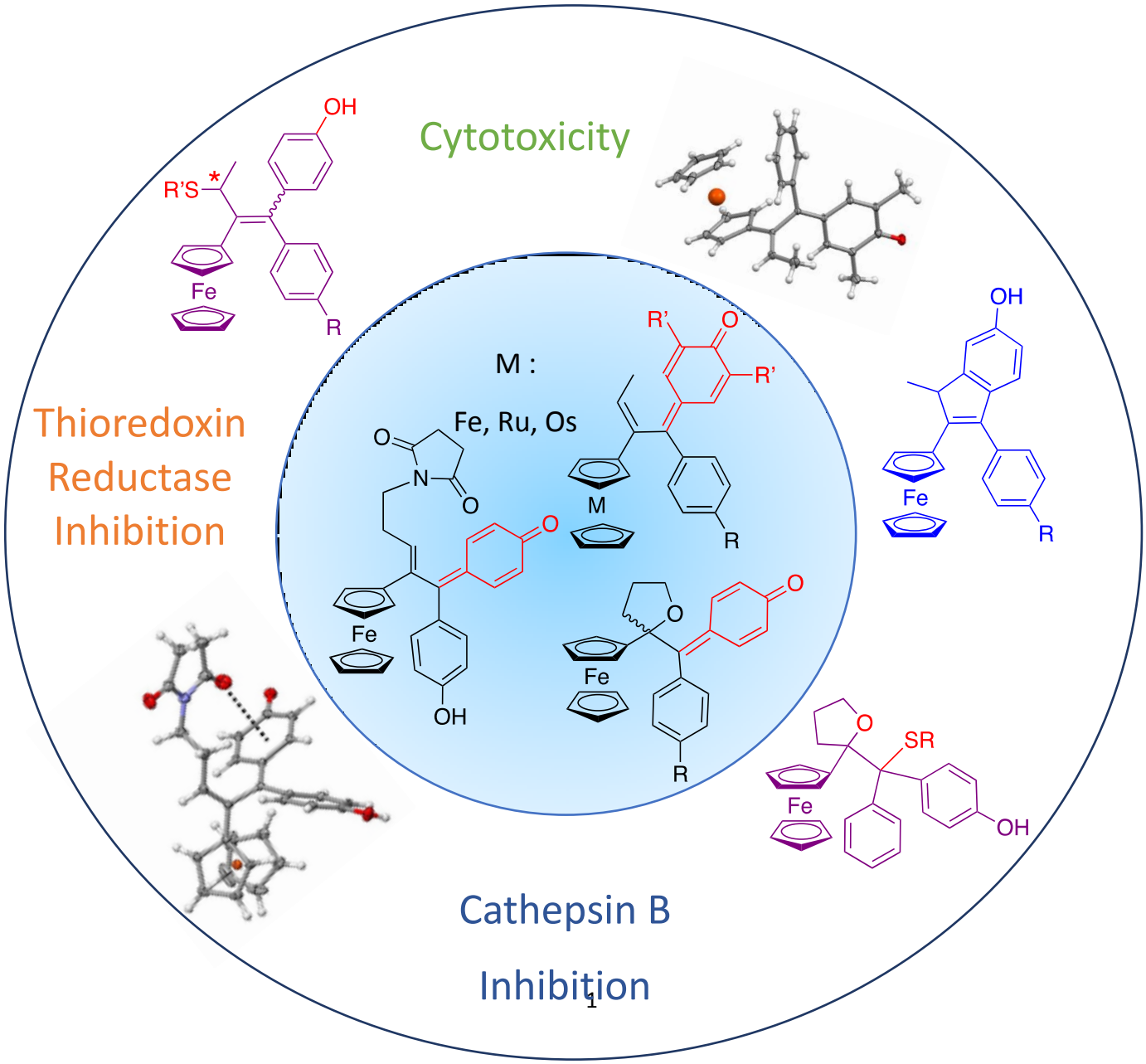


Highlights

- Organometallic quinone methides are more stable than their corresponding organic counterpart allowing their fully characterisation (including X-ray structures)
- The ferrocenyl-ene-phenol motif is the key of the original and diversify reactivity of ferrocenyl quinone methides
- Ferrocenyl quinone methides are able to disrupt redox balance in cancer cells inducing an unprecedented cytotoxicity
- Among the triad, iron, ruthenium, osmium, iron complexes are the most cytotoxic
- Osmium complexes can be precisely localized within the cells

Abstract

Organometallic quinone methides (OM-QMs) have unique chemical and biological properties compared to their purely organic counterparts, but their originality has not previously been delineated in review form. These phenomena are particularly evident when they are incorporated into a judiciously chosen substrate, in this specific case the ferrocifens, bioorganometallic modifications of hydroxytamoxifen, the antiestrogen of reference. The newly created architecture reveals an embedded ferrocenyl-ene-phenol motif that is key to the formation of metallocene quinone methides by reversible oxidation, either chemically, electrochemically or enzymatically, whereby the sandwich unit functions as a redox antenna. In cancer cells, the QMs are primary metabolites that behave as selective electrophiles that undergo Michael additions with thiols or selenols of key proteins crucial to maintaining redox balance, thus generating a disruption of cell metabolism. Within this class of metallocene complexes, the ferrocifens are the most cytotoxic of the iron, ruthenium, osmium triad against a wide range of cancer cells, while osmium allows the complexes to be used as an imaging probe. We describe here the syntheses and structures of ferrocifen derivatives carrying substituted alkyl, imido or hydroxyalkyl chains that allow access to new types of biologically effective quinone methides. The potential of OM-QMs in chemistry and biology is thus demonstrated in its diversity.

Keywords: Bioorganometallic chemistry, cross coupling, Redox chemistry, Iron, Antitumor agents

Contents

1 Introduction

Bioorganometallic chemistry is a multidisciplinary field focused on the bioactivity of molecules with at least one metal-carbon bond [1–6]. This unifying neologism, coined by us in the mid-1980's [7], arose as a response to the need to extend the range of efficacy and mitigate the side-effects of the available metal-based medications, such as the widely-used coordination complexes of platinum. Initially, we chose to focus on potential treatments for breast cancer, which affects one woman in eight in the Western world. However, this continually burgeoning domain now encompasses some aspects of environmental science, toxicology, metallomics, energy, biosensors, radiopharmaceuticals, natural and artificial enzymes, bioanalysis, and medicinal chemistry [8].

2 Bioorganometallics and breast cancer

Following the famous mantra of Sir James Black, "The most fruitful basis for the discovery of a new drug is to start with an old drug" [9,10], our initial approach involved the incorporation of judiciously selected organometallic moieties into hydroxytamoxifen, **1**, the active metabolite of tamoxifen, **2**, the drug classically prescribed to treat hormone-dependent breast cancer [11,12]. Gratifyingly, replacement of the phenyl group adjacent to the ethyl substituent in **1** by a cyclopentadienyl ring bearing an organometallic fragment yielded molecules exhibiting antiproliferative activity on both hormone-dependent and hormone-independent cancer cells. Although a wide range of molecules of this type has been prepared, containing metals such as Mn, Re, Ru, Os, Co, Pt, Ti, or W (Figure 1) [13–20], it has become apparent that ferrocenyl derivatives of hydroxytamoxifen and their corresponding mono and diphenols (**3**-**5**, Scheme 1) now branded as ferrocifen-type molecules [21], exhibited the greatest potential for bioactivity.

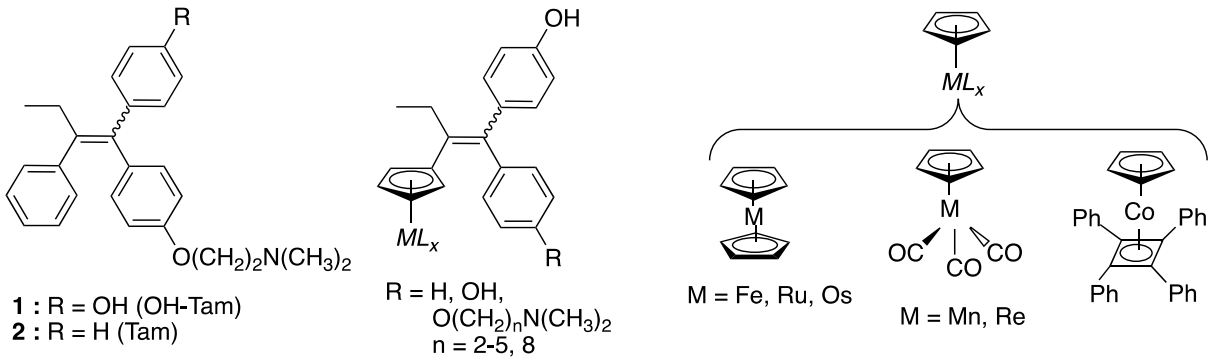


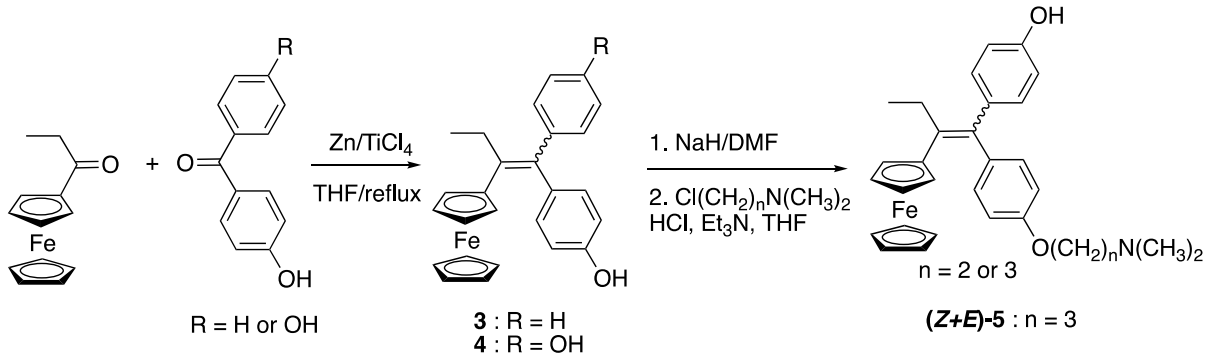
Figure 1. A selection of organometallic fragments incorporated into tamoxifen-type molecules.

The antiproliferativity of the ferrocifens against hormone-dependent breast cancer cells, e.g. MCF-7, may be attributed not only to their structural similarity to tamoxifen, with their dimethylaminoalkoxy chain and ability to bind to the same estradiol receptor site [22], but also by the reversible Fe(II) ↔ Fe(III) redox character of the ferrocenyl moiety that leads to formation of Reactive Oxygen Species (ROS) [23,24]. However, these properties cannot alone account for their remarkable behaviour against hormone-independent cancers, such as MDA-MB-231. It has now been established that the formation of ferrocenyl quinone methides (QMs) plays a crucial role, as we describe herein.

3 Synthetic aspects

3.1 Ferrociphenols and Ferrocifens

As exemplified in Scheme 1, ferrociphenols, **3** and **4**, were readily synthesized by McMurry coupling of the appropriate ferrocenyl ketone and a suitable benzophenone; subsequent incorporation of the dimethylaminoalkyl basic chain furnished the corresponding ferrocifens. We note that the ferrocifens are formed as *E*/*Z* mixtures (generally around 50/50) that interconvert very readily in the presence of acids, even in trace quantities and also in aqueous biological medium, and so are used as such for biological purposes [25].



Scheme 1. Preparation of ferrociphenols and tamoxifen-like ferrocifens.

This approach has since been extended to include systems in which the ethyl substituent has been modified so as to form hydroxyalkyl [26,27] and imidoalkyl derivatives [28,29]. Analogously, ansa ferrocifens, whereby the ethyl substituent has instead been incorporated into a bridge between the cyclopentadienyl rings, were prepared from the corresponding cyclic ketones [30–33]. It is noteworthy that these McMurry couplings also yield pinacolone rearrangement products; typically, [3]ferrocenophanone and 4-hydroxybenzo-phenone gave the ansa-ferrociphenol **6** (62%) and the corresponding pinacolone, **7**, (11%) whose structures are shown in Figure 2 [34].

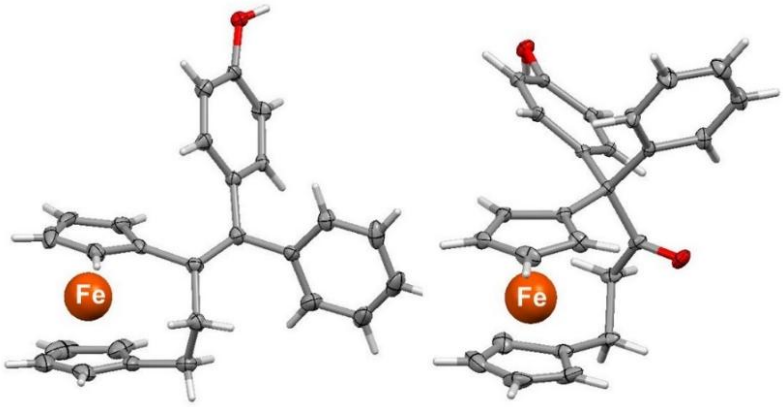
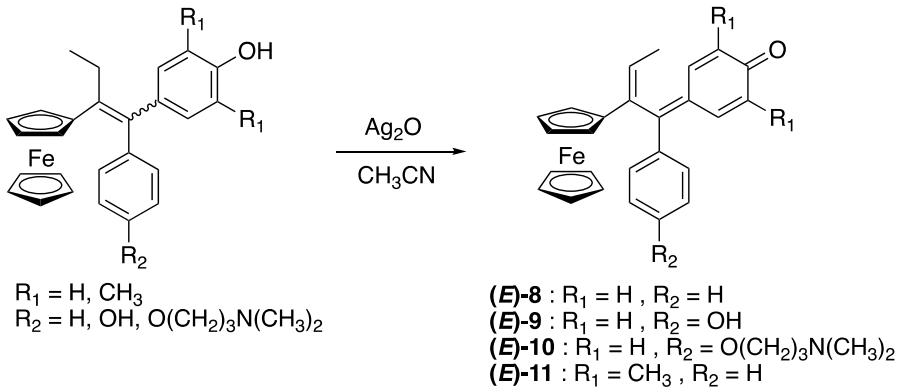


Figure 2. Molecular structures of (left) [3]ferrociphenol, **6**, and (right) its pinacolone rearrangement product, **7**.

3.2 Formation of Quinone Methides by Chemical Oxidation of Ethyl Ferrociphenols

Ferrocenyl QMs **8-11** were first obtained by chemical oxidation of their corresponding ferrocenyl derivatives using silver oxide in acetonitrile (Scheme 2), and were unequivocally characterized spectroscopically (^{1}H and ^{13}C NMR, UV-Vis, IR), and also by mass spectrometry. 2D NMR analysis revealed that only the *E* isomers are obtained [35], an observation further discussed in Section 3.9.



Scheme 2. Chemical oxidation of **3-5** leads to formation of quinone methides **8-11**.

Quinone methides **8** and **10**, obtained from mono-phenolic and tamoxifen-like ferrocifens, can be isolated in pure form as solids and, as such, are stable for at least two months. In contrast, **9**, the QM obtained from the ferrocenyl-diphenol **4** was identifiable by NMR spectroscopy but could not be isolated in its pure form. This can be attributed to the presence of two phenol substituents leading to uncontrollable overoxidation. We emphasise that the ferrocenyl QMs **8** and **10** are significantly more stable than the QMs of their organic counterparts derived from hydroxytamoxifen, **1**, whose stability in biological medium is

limited to 3 – 4 hours [36,37]. Further confirmation of this quinone methide structure was provided by X-ray crystallography for the quinone methide, **11**, in which the carbonyl group derived from the ferrociphenol **12** is flanked by two stabilizing methyl substituents [35]; the alternating long and short bonds in the quinone ring and beyond are illustrated in Figure 3.

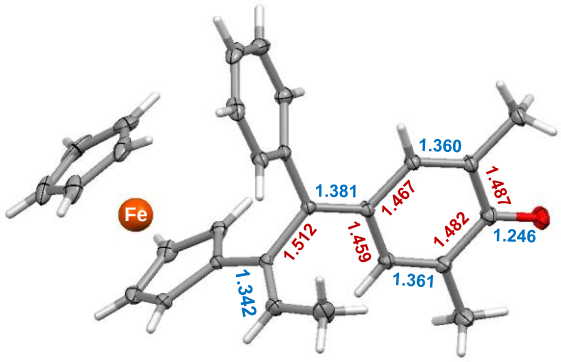
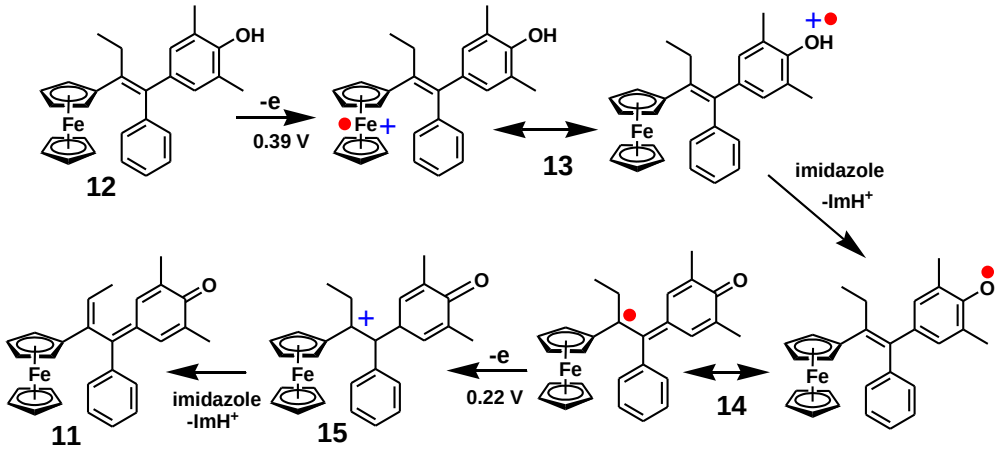


Figure 3. X-ray crystal structure of the ferrocenyl quinone methide ***E*-11**; distances in Å.

3.3 Electrochemical Route to a Quinone Methide

The conversion of a precursor ferrociphenol to a quinone methide requires the overall loss of two hydrogens, and this process was investigated in a cyclic voltammetry/EPR study on the dimethylferrociphenol **12** to form the corresponding QM, **11**, the results of which are summarized in Scheme 3 [38].



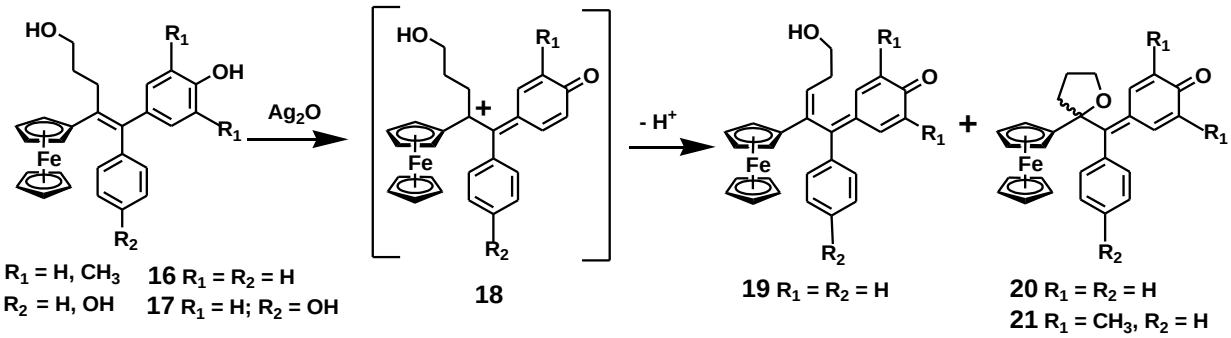
Scheme 3. Stepwise oxidation/deprotonation sequence to form a quinone methide.

In the absence of imidazol, **12** exhibits a reversible one-electron wave at 0.39 V, entirely typical for a ferrocenyl system, corresponding to the oxidation of Fe(II) to Fe(III). Formally this process generates a radical cation, **13**, with a 17-electron configuration at iron; EPR data revealed that the free electron resides primarily at iron, but some delocalization onto

a phenolic oxygen is readily envisaged. Deprotonation using imidazole yields the radical **14** for which EPR data indicate that the unpaired electron density is mostly on the carbon adjacent to the ferrocenyl substituent. A second oxidation (0.22 V) leads to the ferrocenyl-stabilized cation **15**, and a final deprotonation furnishes the observed QM, **11** [38].

3.4 Formation of Quinone Methides by Chemical Oxidation of Hydroxyalkyl-ferrociphenols

The electrochemical data provide a rationale for the products observed upon oxidation of the hydroxypropyl-ferrociphenols **16** and **17** with Ag_2O that each yield two QMs (Scheme 4). The metal-stabilized cationic intermediate **18** (analogous to **15** in Scheme 3) can either undergo deprotonation in the side chain to form **19**, or cyclize to yield the novel tetrahydrofuran **20** (in the ratio 1:9, respectively) [26]. The structure of the corresponding derivative, **21**, in which the carbonyl group is flanked by methyl substituents, was characterized by X-ray crystallography and the envelope conformation of the THF moiety is clearly apparent (Figure 4, right).



Scheme 4. Chemical oxidation of hydroxypropyl-ferrociphenols yields two different QMs.

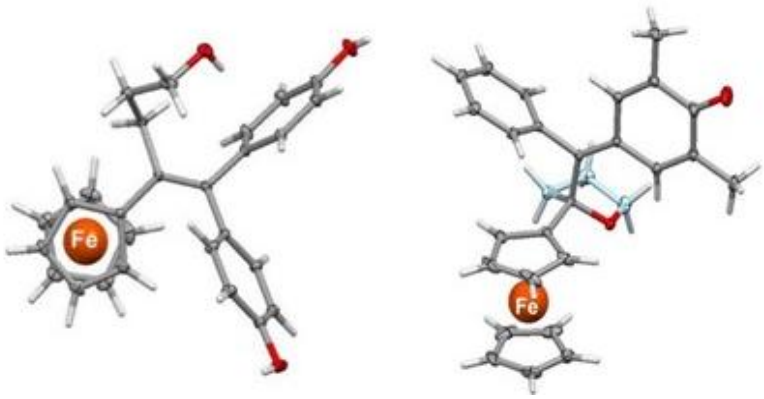
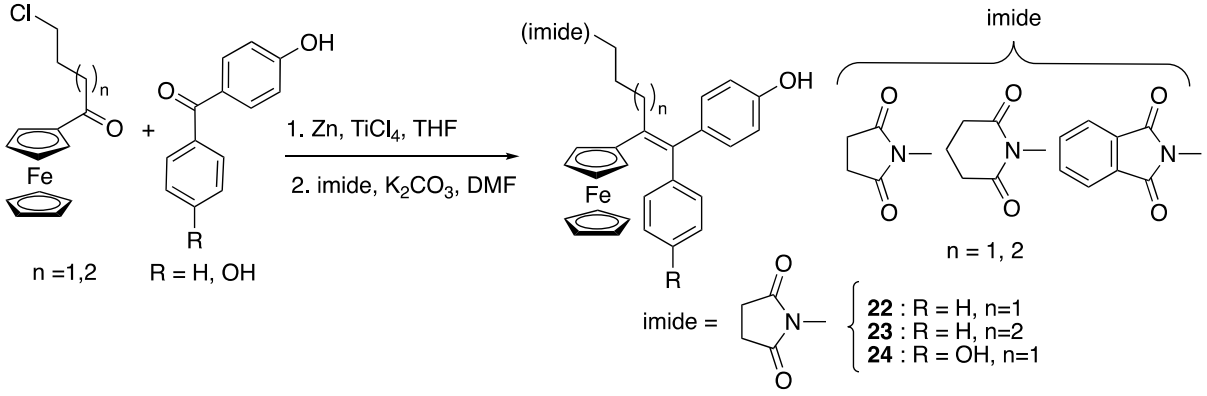


Figure 4. Molecular structures of (left) the hydroxypropyl-ferrocidiphenol, **17**, and (right) the THF-containing ferrocenyl quinone methide, **21**.

3.5 Formation of Quinone Methides by Chemical Oxidation of Imidoalkyl-ferrociphenols

The imidoalkyl-ferrociphenols (imido = succinimido, glutarimido or phthalimido) that were prepared as in Scheme 5 and characterized by X-ray crystallography, were also oxidized by silver oxide to their corresponding quinone methides [28,29]. Typically, in the succinimidopropyl-ferrociphenol, **22**, the imido and aryl ring planes are almost parallel and separated by 3.84 Å (Figure 5, left). This phenomenon also obtains in the glutarimido and phthalimido analogues. We note, however, that such an interaction is viable only in molecules possessing a propyl chain. Figure 5 (right) depicts the situation observed in the analogous succimidobutyl-ferrociphenol **23**, in which the longer chain can no longer fold round so as to lie closely parallel to the adjacent phenol ring, and indeed is directed away from it.



Scheme 5. Preparation of imidopropyl- and imidobutyl-ferrociphenols

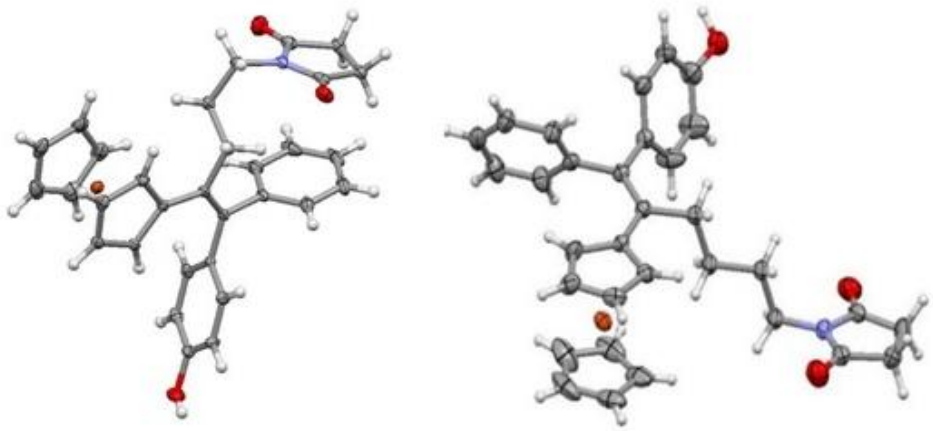
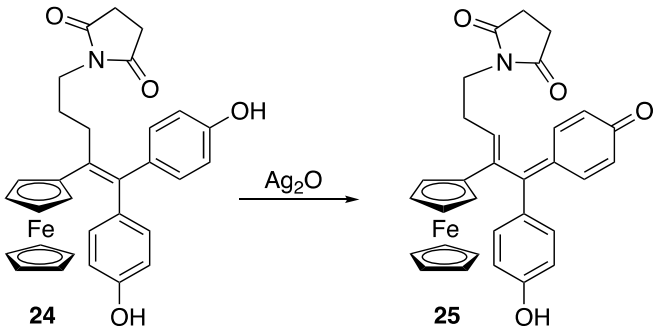


Figure 5. Structures of (left) the imidopropyl-ferrociphenol, **22**, and (right) the imidobutyl-ferrociphenol, **23**, showing the different orientations of the imido group and the aryl ring.

Chemical oxidation of the succinimidopropyl-ferrocidiphenol, **24**, with Ag_2O gave the corresponding quinone methide, **25**, (Scheme 6) that was also characterized X-ray crystallo-

graphically (Figure 6, left). Here the imido group has rotated such that the carbonyl oxygen now lies only 3.25 Å from the aryl ring (i.e. closer than 3.84 Å found between the imido and aryl ring planes in its precursor). The structure of the analogous glutarimidopropyl quinone methide, **26**, also shows this same feature (Figure 6, right) [29]. These molecules, whereby an oxygen of an imido carbonyl is oriented so as to be proximate to a neighbouring aryl ring, provide examples of the increasingly invoked lone pair-π (lp-π) interaction, a phenomenon involving a stabilizing association between a lone pair of electrons and the face of a π system [39]. Although perhaps counterintuitive, and rather weak (1.5 - 2 kcal mol^{-1}), it is now recognized as a new supramolecular bond and has been observed in biological macromolecules [40] and host-guest systems [41].



Scheme 6. Chemical oxidation of the succinimidopropyl-ferrocidiphenol **24** to form QM **25**.

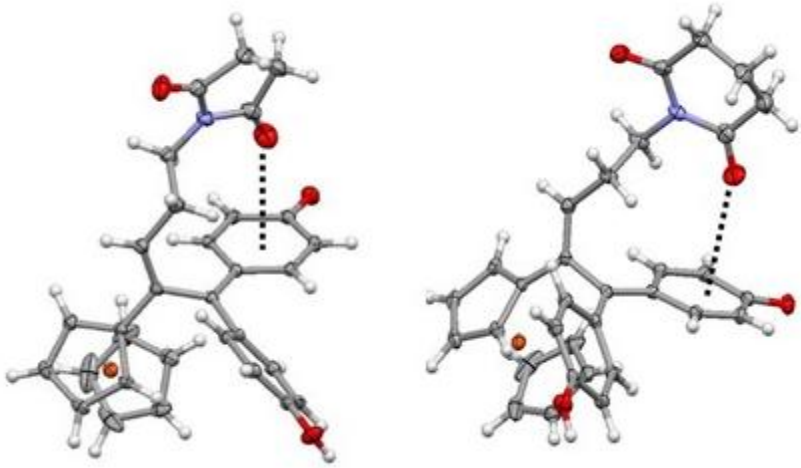


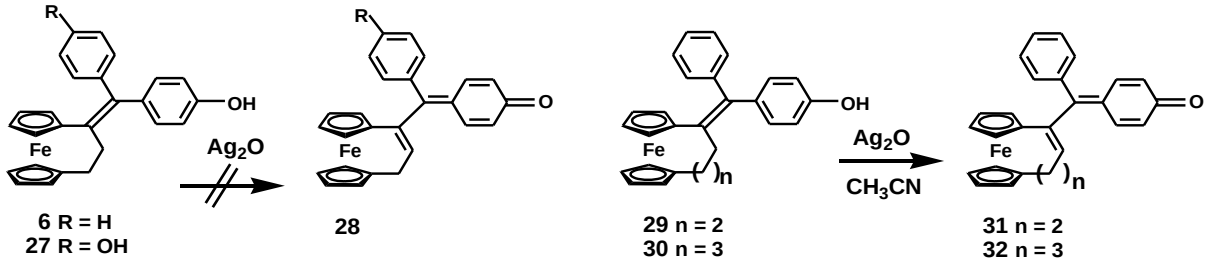
Figure 6. Molecular structures of (left) the succinimidopropyl-ferrocenyl QM, **25**, with a stabilized lone pair-π interaction, and (right) the glutarimido analogue, **26**.

The half-lives of these QMs are markedly lengthened. Typically, at pH 5, the vinyl-ferrocifenyl-QM, **9**, decays to its inactive indene with $t_{1/2}$ around 10 min, whereas for **25** its half-life is now approximately 40 min. For further comparison, the imidoalkyl-ferrocifenyl-

QMs with butyl or pentyl chains can survive in acetone for a few hours before decaying to the corresponding indenes, whereas the imidopropyl systems exhibit half-lives of at least a week before finally forming multiple products [29].

3.6 Chemical Oxidation of Ansa Ferrociphenols

Addition of Ag_2O to a solution of the ansa-ferrociphenols **6** or **27** brought about the characteristic colour change from yellow-orange to red; however, it was not possible to detect the QM product, **28**, by NMR spectroscopy, whereas such is the case for its open-chain analogues **8** and **9**. This may be rationalizable in terms of the ring strain associated with the introduction of a double bond into the shorter bridge. However, when the bridging link was extended to three or four methylenes, as in **29** or **30**, the resulting quinone methides, **31** and **32**, are isolable as stable solids (Scheme 7) [30].

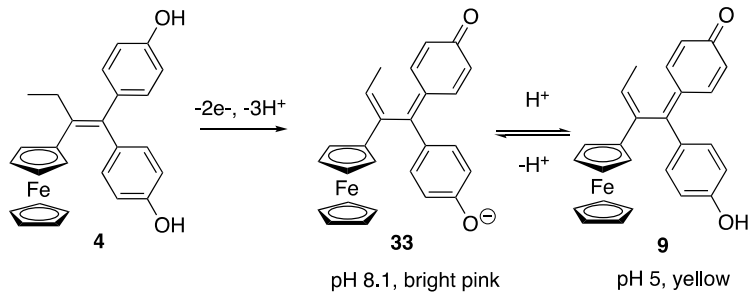


Scheme 7. Chemical oxidation of ansa-ferrocifens yielded QMs only for n = 2 or 3.

3.7 Formation of Quinone Methides by Enzymatic Oxidation Using HRP/H_2O_2

Quinone methides can also be obtained from ferrociphenols when incubated with the system horseradish peroxidase/H_2O_2 (HRP/H_2O_2) in a phosphate buffer at pH 8.1. Typically, tamoxifen-like ferrocifen **5** and HRP/H_2O_2 yield the QM **10**, previously obtained by chemical oxidation with silver oxide (Scheme 2). This oxidation is easily followed by UV-Vis spectroscopy thanks to the strong absorbance of **10** at 402 nm. The reaction is fast (k = 0.4 min^{-1}) and the product is stable in biological media [42,43]. Analogously, enzymatic oxidation of the ferrociphenol **3** furnishes the corresponding quinone methide **8**.

Under the same conditions (pH 8.1), the ferrocidiphenol **4,** yields the phenolate quinone methide **33**, characterized by its bright-pink colour, while at pH 5 the yellow neutral QM, **9**, is obtained (Scheme 8, Figure 7) [44]. This bright pink colour is associated with conjugation analogous to that found in the pH indicator phenol red. As noticed previously for its chemical oxidation with Ag_2O, this QM is quite unstable ($t_{½}$ = 12 min at pH 8.1).



Scheme 8. Enzymatic oxidation of ferrocidiphenol **4** with HRP/H_2O_2 yields, at pH 8.1, the QM in its phenolic form, **33**, and in its neutral form, **9**, at pH 5.

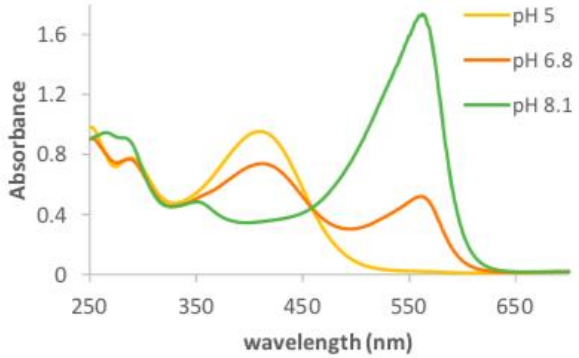
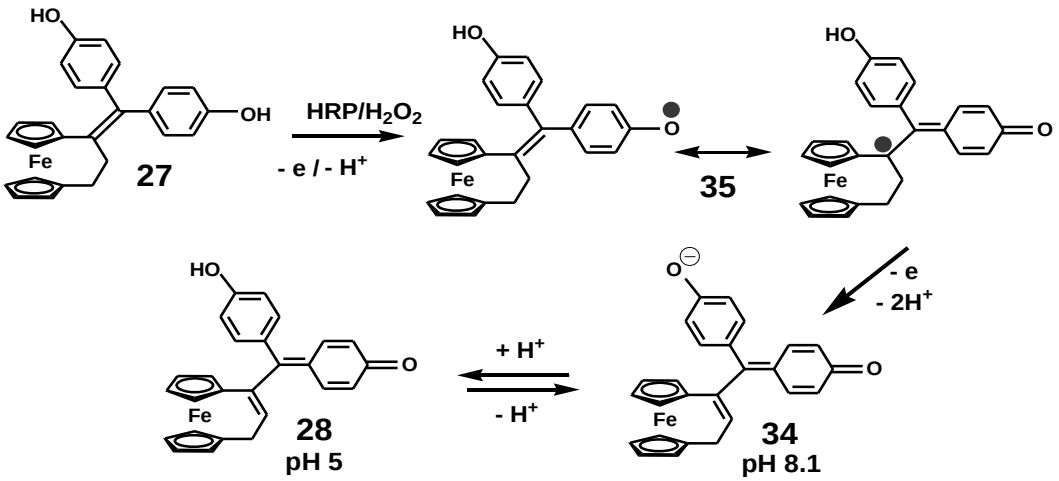


Figure 7. UV-Vis spectrum of ferrocidiphenol **4** after 2 min of incubation with HRP/H_2O_2 at different pH values: phenolate **33**, 553 nm at pH 8.1; neutral phenol **9**, 406 nm at pH 5.

Of particular interest, however, is the behaviour of the ansa-ferrocidiphenol, **27**, when compared to that of its open-chain counterpart, **4**. Analogous to the behaviour observed earlier with **4**, enzymatic oxidation of **27** leads to **34**, the QM in its phenolate form at pH 8.1, and to **28**, the QM in its neutral form at pH 5 (Scheme 9) [45]. However, the rate of formation of **34** at pH 8.1 (k = 2.5 min^{-1}) is much higher than for the open-chain system **9** (k = 0.12 min^{-1}); this may be rationalized in terms of the ring strain caused by the introduction of a double bond in the bridge (Scheme 7). Indeed, UV-Vis and EPR data supported by calculations at the DFT level reveal that the delocalized radical **35** has a short but detectable lifetime [46]. This may be a consequence of the limited ability of the ansa-ferrocenyl moiety to stabilize the cation analogous to **15** in Scheme 3. Interestingly, as shown earlier in Scheme 7, chemical oxidation of **27** failed to yield the ansa-QM **28**; this is in accordance with the low stability observed for the QM-phenolate **34**, since gradual conversion of its pink colour to brownish was observed after 105 min with the appearance of insoluble species.

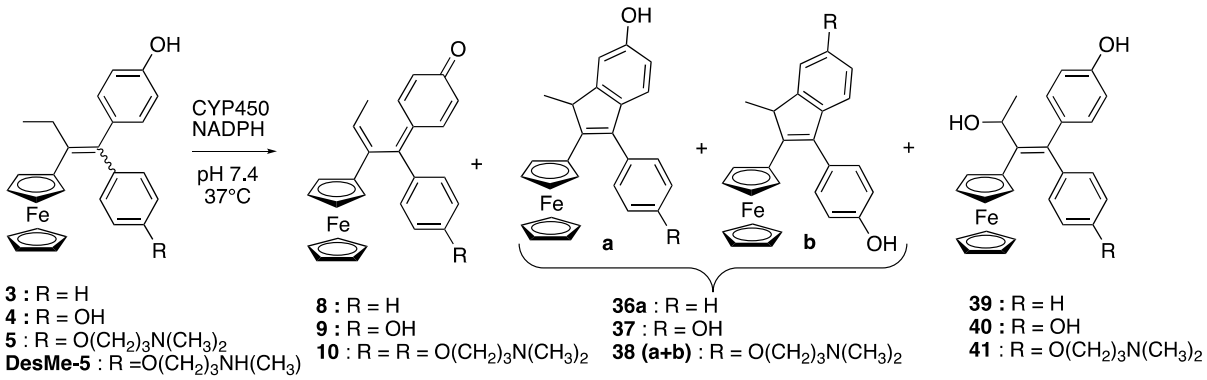


Scheme 9. Intermediacy of the short-lived, but detectable, ansa-QM radical **35**.

3.8 Formation of Quinone Methides by Enzymatic Oxidation Using CYP450

Cytochromes P450 (CYP450) are a family of enzymes that play a major role in drug metabolism, accounting for about 75% of total metabolism. Thus, an *in vitro* study of the oxidation of molecules with CYP450 gives a preliminary indication of their *in vivo* metabolism.

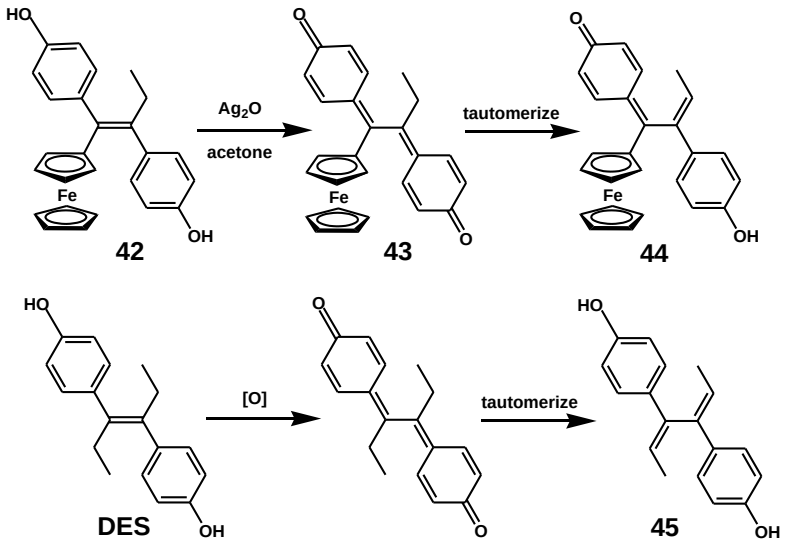
When the complexes **3**-**5** were incubated in a phosphate buffer (pH 7.4) with a suspension of liver microsomes, used as a source of CYP450, containing NADPH (nicotinamide adenine dinucleotide phosphate) at 37°C, they gave, in each case three metabolites (Scheme 10): their QMs, **8**-**10**, and the corresponding indenes **36**-**38**, resulting from the transformation of QMs in a protic medium (see Section 4.1); the corresponding allylic alcohols **39**-**41** were also formed [47]. The tamoxifen-like ferrocifen **5** yielded metabolic products that retained the aminoalkoxy basic chain (**38a** and **38b**), as well as the diphenol **4**, and **DesMe**-**5**.



Scheme 10. Formation of the three main metabolites from phenols **3-5**. **DesMe-5** is an additional metabolite of **5**.

3.9 Importance of the Ferrocenyl-ene-p-phenol Motif

As revealed by the electrochemical study of the conversion of a ferrociphenol to a QM, the redox-active ferrocenyl substituent plays a key role, not only as an intramolecular reversible redox ″antenna" but also as a stabilised carbocation ″modulator". We emphasise, however, that the particular structural motif *ferrocenyl-ene-p-phenol* [48,49] and the *E geometry* of the system are crucial for the generation of antiproliferative quinone methides. A change in position of either the OH or the ferrocenyl group leads to diminished cytotoxicity. For example, in **42** whereby the ethyl and phenol substituents have exchanged positions relative to that in the ferrocifens, chemical oxidation yields initially the rather unstable blue-green diquinone, **43**, that was characterized only by NMR spectroscopy; eventually, however, tautomerization yields the stable green phenol-quinone, **44** (Scheme 11). Most importantly, however, cyclic voltammetry revealed successive oxidation waves at 0.4 V and at 0.8 V clearly demonstrating that **42** does not follow the behaviour of ferrociphenol **27**, but instead resembles that of diethylstilbestrol (DES) which also yields a diquinone before rearranging into the diphenol **45** [50].

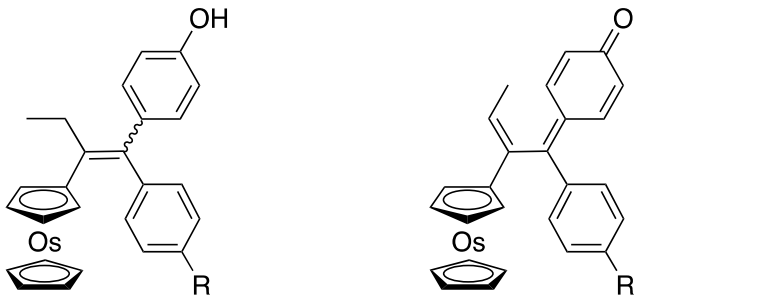


Scheme 11. Oxidation of ferrociphenol **42**, via diquinone **43**, to **44**.

A possible rationale for the exclusive formation of *E* quinone methides invokes the preference for an almost coplanar, orientation of a cyclopentadienyl ring with the central double bond and the phenol so as to optimize π overlap, and facilitate delocalization of the initially generated radical cation, as depicted in Scheme 3. A planar transition state with *Z* geometry would undoubtedly engender steric problems between the ferrocenyl moiety and the adjacent phenolic ring [44].

3.10 Formation of Quinone Methides Derived from Osmocene

Ferrocifen-type derivatives of ruthenocene and osmocene, the heavier metallocenes of the iron triad, have both been studied, but more focus has been placed on the latter [20,43,45] Osmium complexes **46**-**48** (Figure 8) were prepared via the analogous McMurry cross-coupling of propionylosmocene with the appropriate benzophenone, but require higher temperatures and a stronger acylating agent, and the yields are lower (40-50%; quantitative for Fe [20,22]. Osmium, unlike iron, is a heavy metal not present in the body and, as such, is a very good marker for imaging studies of cells by using techniques such as X-ray spectrofluorescence. Indeed, it has been used to determine the intracellular distribution of hydroxyosmocifen, **48** in MDA-MB-231 cell [51].



46 : **R =** H
47 : R = OH
48 : R = $O(CH_2)_3N(CH_3)_2$

49 : **R =** H
50 : R = OH
51 : R = $O(CH_2)_3N(CH_3)_2$

Figure 8. Osmocifens and their corresponding quinone methides.

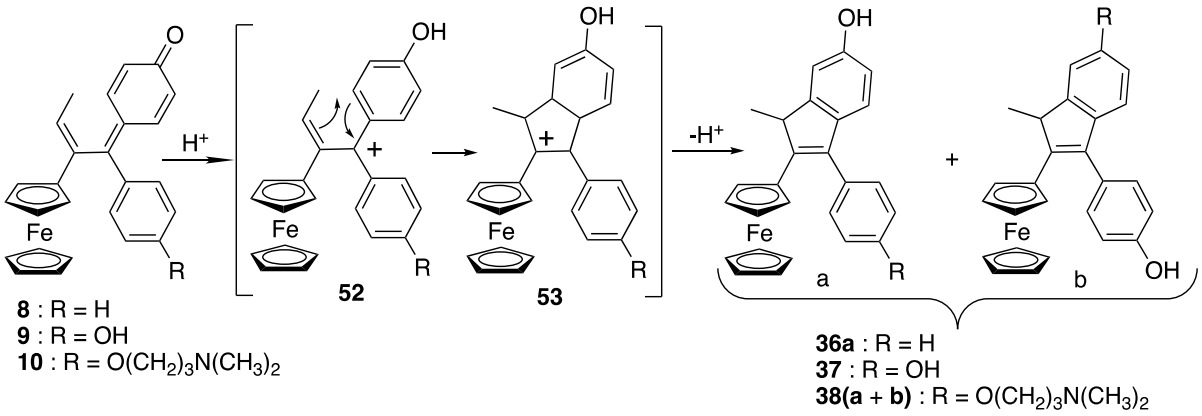
Oxidation of **46**-**48** by Ag_2O furnished the corresponding quinone methides, **49**-**51**. However, the rates of formation for the osmium complexes are significantly lower than those of the corresponding iron ones (0.48 $x10^{-3}$ s^{-1} for **51** versus 1.6 x 10^{-3} s^{-1} for **10**) [20]. In contrast, enzymatic oxidation of osmium complexes by the HRP/H_2O_2 system is different from that of their iron counterparts. In the case of **46** and **48**, the oxidation process stops at the carbenium ion precursors (analogous to **15** in Scheme 3) of the quinone methides; moreover, these carbocations can be precipitated by the addition of $NaBPh_4$ and characterized by their UV-Vis and mass spectra [45]. Failure to obtain the neutral quinone methide is rationalizable in terms of the greater stabilization of the osmocenyl carbocation relative to that of the corresponding ferrocenyl system, presumably because of better overlap between the vacant *p* orbital on carbon with a filled *d* orbital on the metal [52]. Electrochemical oxidation of the osmium complexes, **46** and **48** led to irreversible oxidation without the need for addition of base [53], whereas this is necessary for their ferrociphenyl counterparts [21]. This indicates a clear difference in acidity of the phenol in the iron and osmium complexes.

4 Chemical Reactivity of Ferrocifenyl Quinone Methides

Quinone methides (QMs) are normally encountered as short-lived intermediates but the transient stability of these species can be modulated depending on substituents [54]. These organic species are fairly powerful electrophiles obtained chemically by oxidation of alkyl phenols, such as **1**, with Ag_2O or MnO_2. They are likely to be of interest in oncology [55] through their addition reactions with nucleophiles present in biological media (DNA, proteins) [56].

4.1 Protonation of Quinone Methides Derived from Ethyl Ferrociphenols

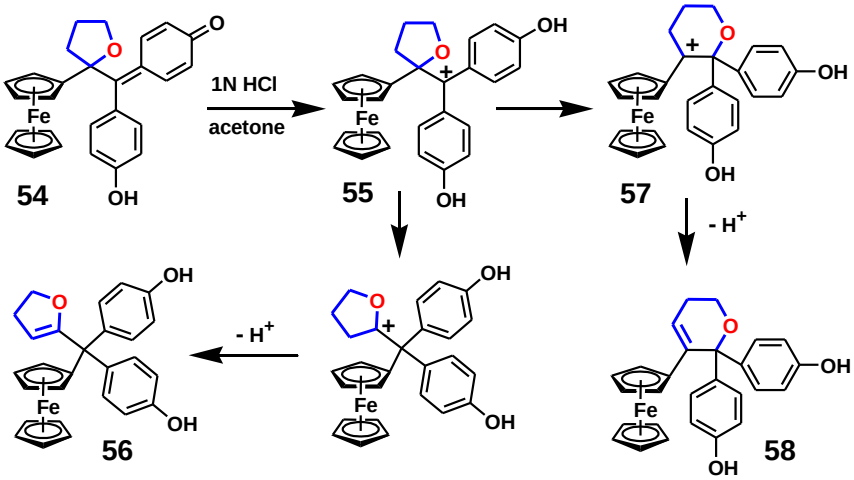
In acidic media (1N HCl in acetone), or in the presence of an electrophile such as $ZnCl_2$, the QMs **8**-**10**, are transformed into indenes **36**-**38** (Scheme 12). Protonation generates the cation **52** which undergoes cyclization to form the ferrocenyl-stabilized species **53**, and ultimately yields the indenes, that are more stable than their corresponding QMs. For **10**, the tamoxifen-like QM, a mixture of indenes **38a** and **38b** (ratio 7:3) is obtained [47].



Scheme 12. Rearrangement of QMs into indenes upon protonation.

4.2 Protonation of a Tetrahydrofuranyl-ferrocifen Quinone Methide

Protonation of the tetrahydrofuranyl-QM **54** by HCl in acetone generates the doubly benzylic carbocation, **55**, that can undergo two molecular rearrangements (Scheme 13). Migration of the ferrocenyl substituent and subsequent loss of a proton yields the dihydrofuran **56**, whereas ring expansion to form **57** furnishes the dihydropyran **58**, both of which were unambiguously characterized by X-ray crystallography (Figure 9) [26,57].

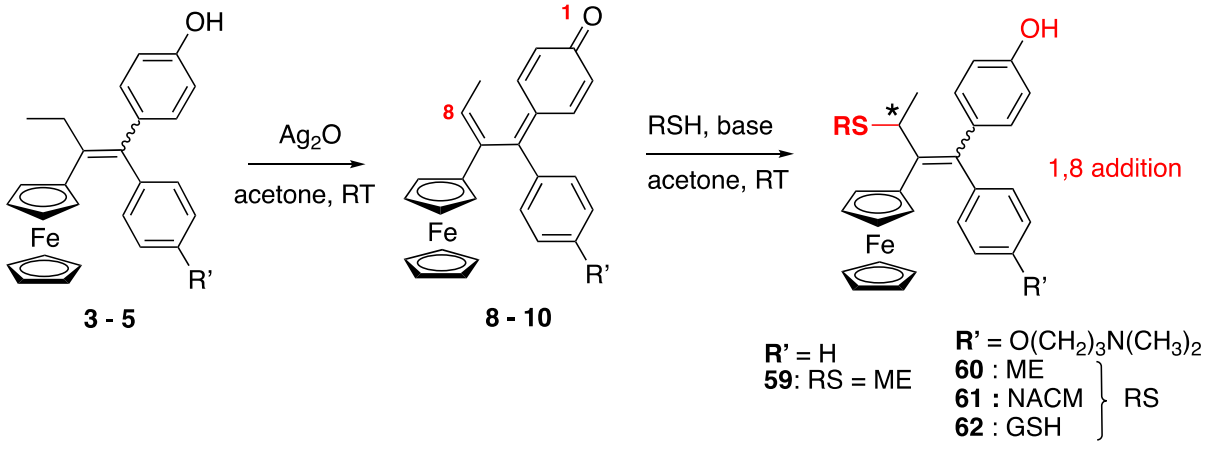


Scheme 13. Rearrangement of a protonated THF-quinone methide.

Figure 9. Molecular structures of (left) the dihydrofuran **56** and (right) the dihydropyran **58**.

4.3 Reactions of Ferrocifenyl-QMs with Thiols

The susceptibility of QMs to attack by nucleophiles, such as peptides or proteins bearing thiols or selenols, could lead to cell death by interference with oxidative stress or inactivation of enzymes. Accordingly, generation of QMs by chemical oxidation with Ag_2O followed by reaction with a range of thiols – mercaptoethanol (ME), *N*-acetyl-*L*-cysteine methyl ester (NACM), glutathione (GSH) – yielded 1,8-Michael adducts **59**-**62** (Scheme 14), that were fully characterized spectroscopically or by X-ray crystallography [58], as typified by the mercaptoethanol adduct, **59**, shown in Figure 10.



Scheme 14. 1,8-Michael additions of thiols, mercaptoethanol (ME), glutathione (GSH), *N*-acetyl-*L*-cysteine methyl ester (NACM), to a vinyl-ferrocifenyl-QM.

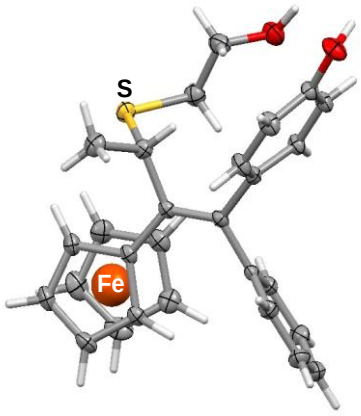
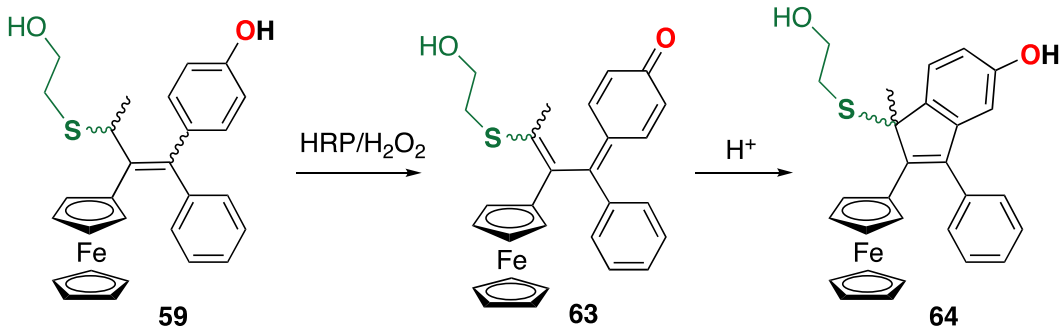


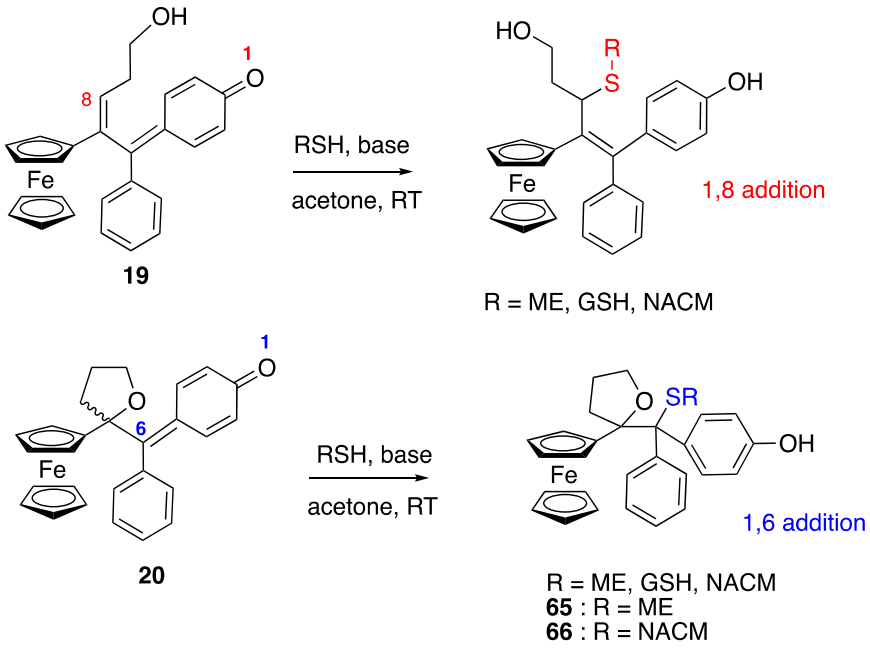
Figure 10. Molecular structure of the 1,8-mercaptoethanol adduct **59.**

Likewise, generation of these QMs by incubation of ferrociphenols with rat liver microsomes in the presence of NADPH, and subsequent reaction with thiols, furnished the same products. Interestingly, incubation of thiol 1,8-adducts, such as **59,** with the mixture horseradish peroxidase/H_2O_2 led to further oxidation resulting in regeneration of the quinone methide skeleton, now containing a thiol substituent, as in **63** (Scheme 15) [58]; subsequent protonation brought about cyclization to form the corresponding indene **64** – a known reaction of cations of this type (see Scheme 12) [59].



Scheme 15. Enzymatic oxidation of a ferrocifen-QM—thiol adduct, ultimately forming an indene.

The two types of QM, **19** and **20**, derived from the hydroxypropyl-ferrociphenol **16** are also susceptible to nucleophilic attack by alcohols or thiols. As depicted in Scheme 16, reaction of **19**, which possesses a hydroxypropenyl sidechain, with thiols or alcohols yields 1,8-adducts thus paralleling the behaviour seen previously with the vinyl system (Scheme 14). In contrast, however, the THF isomer, **20**, forms 1,6-adducts such as **65** and **66** [57].



Scheme 16. 1,6- and 1,8-Michael additions of thiols to ferrocifenyl quinone methides.

4.4. Reactions of Ferrocifenyl-QMs with Thioredoxin Reductase

Thioredoxins (Trx), are small (12 kD) proteins that are ubiquitous in cells, and which act as antioxidants. They possess vicinal cysteines that undergo intramolecular dithiol—disulfide exchange when they react with ROS; for example, with hydroxyl they form water and sulfur-based radicals that couple to form the corresponding disulfide. Recovery of the dithiol structure is catalysed only by thioredoxin reductase (TrxR) in conjunction with NADPH. Thioredoxin reductases (TrxRs) are enzymes which, together with the glutathione system, are responsible for maintaining thiol redox balance in cells [60]. TrxRs possess an N-terminal redox center characterized by a dithiol motif (Cys-XXXX-Cys) and a C-terminal active site with a -Gly-Cys-Sec-Gly motif (Sec = selenocysteine). The Sec residue is characterized by a low pKa (5.24 for the selenol/selenate couple compared to 8.25 for the thiol/thiolate couple) resulting in enhanced nucleophilic character [61]. TrxRs are often overexpressed in cancer cells and their inhibition brings about cell death [62]. Consequently, molecules able to inhibit TrxRs are considered as potential anti-cancer drugs and have been extensively studied recently [63], in particular with ferrocifens, osmocifens and their corresponding quinone methides.

Initial results have been obtained for **3** and **5**, and their QMs, **8** and **10**, respectively, that were produced by chemical oxidation (Scheme 2) since their stability is sufficient to allow them to be isolated. The data presented in Figure 11 revealed that both QMs are good

inhibitors of TrxR (IC_{50} = 2.6 and 2.2 µM) while their precursors are significantly less so (IC_{50} ~15 µM) [42].

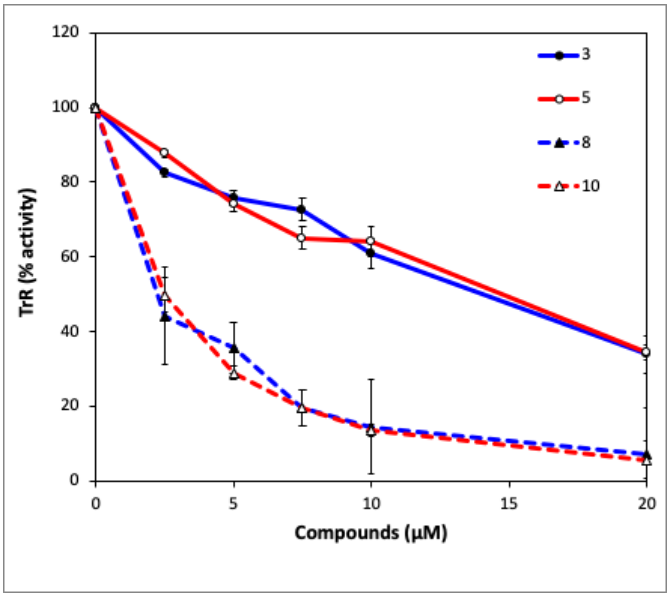


Figure 11. Thioredoxin reductase (TrxR1) inhibition by ferrociphenol **3** and hydroxyferrocifen **5**, and by their QMs **8** and **10**, respectively, when generated by chemical oxidation.

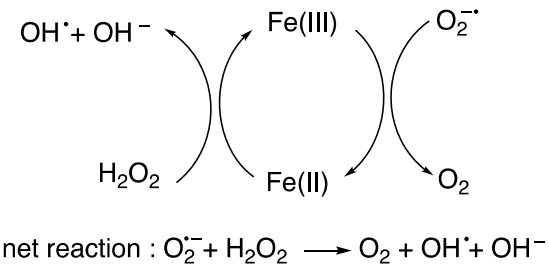
Other experiments have been carried out comprising *in situ* generation of QMs by enzymatic oxidation of ferrocenyl and osmocenyl precursors (**4**, **5**, **27**, **42, 46**, **48)**. We again found that all ferrocifen QMs are very effective inhibitors of TrxR (IC_{50} in the range 0.03 - 0.15 µM) much better than their precursors (8 µM <IC_{50} <32 µM) [43,44,46]. These IC_{50} values found for the QMs are similar to those found for gold complexes which are considered to be very good inhibitors of TrxR [61,62]. The IC_{50} values found for the osmium QMs **49** and **51** are higher (IC_{50} = 3.6 and 5.4 µM) [45], whereas the purely organic quinone methide derived from hydroxytamoxifen **1**, is the least effective molecule in the series (IC_{50} = 8.7 µM) [44].

These results revealed that QM **8** and **10** give adducts resulting from 1,8 Michael addition on the selenol residue Sec498 at the C-terminus of TrxR [42], whereas with QM **28** (from ansa-diphenol) the Cys and Sec residues of TrxR are both alkylated [46].

5 Production and Bioactivity of Reactive Oxygen Species (ROS)

The biological activities of iron in cells are essentially associated with its reversible transformation from Fe(II) to Fe(III) mediated by the Fenton and Haber-Weiss reactions [64].

This catalytic transformation cycle, shown in Scheme 17, is accompanied by the production of ROS known for their bioreactivity.

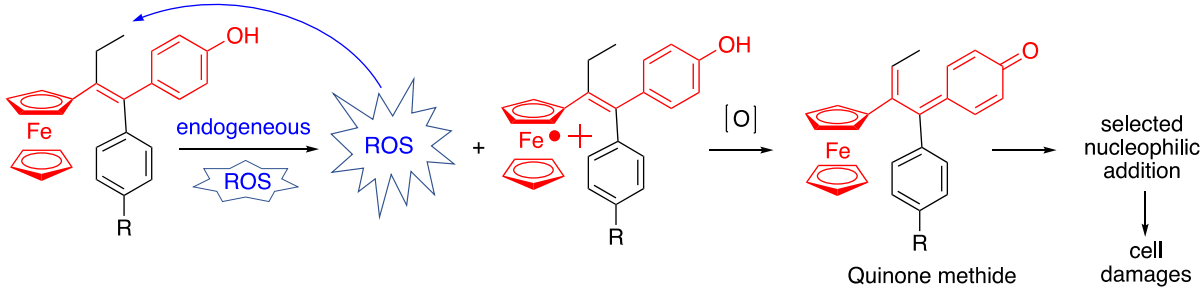


Scheme 17. The Fenton and Haber-Weiss reactions.

The involvement of ROS, in particular the hydroxyl radical, in the rather weak cytotoxicity of ferrocenium salts has been demonstrated by Osella [65]. Subsequently, ROS production was measured, in MDA-MB-231 cells, for a representative selection of ferrocifens and revealed that they all generate significant ROS production after 10 min incubation [24,66]. It was found that the tamoxifen-like complex **5**, and **67** the complex with no phenol function (Scheme 19), are those producing the highest quantity of ROS, thus demonstrating that ROS production does not require the presence of a phenol. Whereas, with ferrocene or hydroxytamoxifen the yield of intracellular ROS is less than that of the controls [66]. The production of ROS is essential for expression of the cytotoxicity of the complexes. This is clearly demonstrated by the observation that addition of antioxidants, such as N-acetyl cysteine or ascorbic acid, causes a marked decrease in the antiproliferativity of ferrocifens (**4**, **5, 17)** on cancer cells [44,67,68]. This quantification made for short incubation times (10 - 120 min) measures the production of ROS associated with oxidative stress following the entry of the complexes into the cells, and is different from that recorded after 24 hours of incubation, which is associated with depolarization of the mitochondrial membranes [24].

It is the base level of ROS in cancer cells that initiates the oxidation of Fe(II) to Fe(III), thereby triggering the sequence of reactions leading to the stepwise synthesis of quinone methides illustrated in Scheme 18. Actually, the ROS generated in cancer cells by ferrocifens and ferrociphenols could in turn interact with them and cause cell death or inactivation by creating organometallic quinone methides that interact with selected nucleophiles or evolve toward other bioactive species. The formation of QMs therefore appears to enhance the cytotoxic potential of ROS, which themselves, at certain concentrations and in certain cell lines, are toxic to the cell. To describe this novel effect, we

propose the term *kronatropic*, an adjective formed from Kronos the Greek deity who devoured his offspring, and Atropos (the eldest of the three Fates) who cuts the thread of life [69].



Scheme 18. Illustration of the kronatropic effect in cancer cells.

The involvement of redox processes and ROS in the cytotoxicity of numerous platinum, gold, ruthenium and rhodium metallodrugs has also been extensively studied [70]. We note, however, that in those cases activation is achieved by a reduction process, for example Pt(IV) to Pt(II) [71], while in the ferrocifens it proceeds by oxidation of Fe(II) to Fe(III)

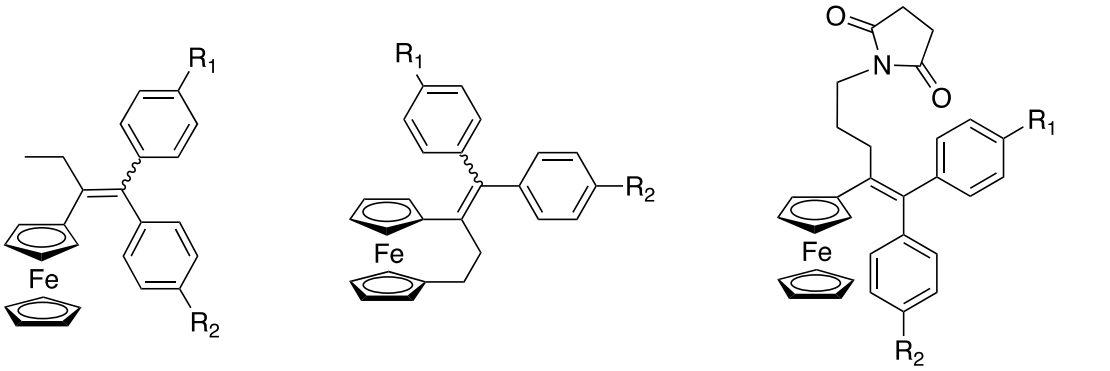
6 Bioactivity of ferrocifenyl quinone methides

6.1 Antiproliferativity of Ferrocifenyl Quinone Methides

The IC_{50} values on cancer cells (MDA-MB-231) have been determined for most of the complexes described here (Table S1) for example for the stable QMs **8**, **10** and **25**, derived from the ferrociphenol **3**, the tamoxifen-type ferrocifen **5**, and the succinimido-propyl-ferrocidiphenol **24**. These values (7.2, 1.8, and 0.56 µM respectively) were found to be 4 to 16 times higher than those of their precursors (1.5, 0.5, and 0.035 µM, respectively) [47]. This result may appear surprising since quinone methides are considered to be involved in the toxicity of complexes *in cellulo*. However, one must appreciate that quinone methides are highly reactive species and are more likely to reach their targets when they are synthesized *in cellulo* than when they have to cross the cell membrane and the cytosol before joining the lipophilic compartments of the cell (nuclei, endoplasmic reticulum, mitochondria, etc.) in which they appear to exert their effect [43,44,51].

6.2 The Role of Quinone Methides in the Cytotoxicity of Ferrocifens

Comparison of the cytotoxicity of three complexes in each of the three series (A-C) bearing zero, one or two phenol groups underlines the role played by the formation of QMs in the cytotoxicity of ferrocifens (Scheme 19).



Series A
4 : $R_1 = R_2 = OH$ [0.6]
3 : $R_1 = OH$, $R_2 = H$ [1.1]
67 : $R_1 = R_2 = H$ [7.6]

Series B
27 : $R_1 = R_2 = OH$ [0.09]
6 : $R_1 = OH$, $R_2 = H$ [0.47]
68: $R_1 = R_2 = H$ [0.92]

Series C
24 : $R_1 = R_2 = OH$ [0.035]
22 : $R_1 = OH$, $R_2 = H$ [0.035]
69 : $R_1 = R_2 = H$ [0.79]

Scheme 19. Series of ferrocenyl complexes with zero, one or two OH groups. IC_{50} values (µM) on MDA-MB-231 shown in square brackets are from Table S1.

Indeed, the complexes bearing one or two phenols (**3**, **4**, **6, 22, 24**, **27**) able to form QMs, are significantly more cytotoxic than their counterparts with no phenol (**67** - **69)**; for the three series, the ratios of the IC_{50} values of complexes with two phenols or no phenol are 13, 10, and 22, respectively. In series C, the same high cytotoxicity is observed for the mono and di-phenolic complexes **22** and **24** (IC_{50} = 0.035 µM). This can be rationalized by the fact that, even though relatively weak, the lone-pair interaction with the quinone methide helps to stabilize its *E* conformation [29]. For the same reason, very similar IC_{50} values are also found for the analogous mono and diphenolic glutarimido complexes (IC_{50} = 0.09 and 0.07 µM) [29]. The factor of two observed between the IC_{50} values of the di- and monophenol complexes of series A and B underlines the importance, for the cytotoxicity of the complexes, of the trans configuration in the ferrocenyl-double bond-phenol motif. This arrangement is present in all the diphenol complexes (**4** and **27**) but in only half of the mono-phenol complexes (**3** and **6**) which are a 50/50 mixture of trans (active) and cis (inactive) species, thus accounting for their lower cytotoxicity [44].

All the ferrocenyl QMs studied are capable of very effectively inhibiting purified thioredoxin reductases *in vitro*. They function via Michael addition to the QM by the

selenocysteine, in some cases also by the cysteine, located in the active site of TrxR, thus counteracting the ability of the cell to defend itself against attack by ROS. However, it is difficult to establish a correlation between the *in vitro* inhibition efficiency of TrxR and the cytotoxicity of the products on cancer cells. This is particularly highlighted in the case of the ansa ferrocidiphenol, **27**, which is one of the most cytotoxic complexes on cancer cells (IC_{50} = 0.089 µM), whereas its quinone methide, **28**, is only an average inhibitor of TrxR (IC_{50} = 0.15 µM). This lack of correlation has previously been found in other systems, such as NHC-gold complexes [72,73]. In addition, it has been shown that, in cancer cells, **8** the QM derived from the monophenol complex **3**, is transformed into the indene **36a** via the rearrangement process depicted in Scheme 12 [42]. These indenes are unable to inhibit TrxR via Michael addition. However, we have recently demonstrated that this indene **36a** is an inhibitor of cathepsin B [74], a protease overexpressed in cancer cells [75], thus becoming a possible target for ferrocifens. Other targets have been previously identified, for example, the estrogen receptor, transcription factors such as AP1 and cytokines [76]. However, the nature of the molecular interactions associated with these mechanisms, thus the possible involvement of QMs, has not been established.

6.3 In vivo studies

The transition between *in vitro* and *in vivo* studies still represents an important challenge in the development of a molecule, and is particularly true in the case of ferrocifens, the administration of which *in vivo* must overcome two specific problems. Their high lipophilicity is an advantage during *in vitro* tests since it allows molecules to easily cross the cell membrane, itself lipophilic, but becomes problematic *in vivo*. The low solubility of the ferrocifens in physiological environments prevents their administration by the intravenous route frequently used for anticancer drugs; furthermore, the phenol, whose half-life in the bloodstream is very short, is very quickly eliminated by the body.

To overcome these difficulties, two types of lipid nanocapsule (LNC) formulation have been developed [67,77] : one consists of a lipid core (oil or triglycerides) in which the active principle is soluble, surrounded by a lipid surfactant (Lipoïd®) and a layer of PEG-HS (functionalised polyethylene glycol) which provides the interface between the lipid phase and the aqueous phase. The LNCs thus obtained can be injected into physiological saline; moreover, judicious choice of PEG makes it possible to obtain stealth LNCs which are not detected by macrophages and thus remain longer in the bloodstream [76,78].

The first *in vivo* results on rat glioblastoma 9L cells (brain cancer) by intravenous injection of LNCs of the ferrocidiphenol **4** led to a 75-80% reduction in size of tumours implanted in rat flank (ectopic tumours) [77]. Addition of a second PEG (DSPE-mPEG2000) to increase the stealth and efficiency of LNCs brought about the virtual disappearance of tumours after intravenous injection of **4**, or the ansa-hydroxyferrocifen, **27** [79,80]. When MDA-MB-231 breast cancer cells implanted in nude mice were treated with stealth nanocapsules loaded with the tamoxifen-like ferrocifen **5**, significant tumour decrease was observed [78]. The combination of radiotherapy, and LNCs loaded with **4**, on 9L orthotopic brain tumours in rats led to increased survival from 25 days in untreated rats, to 40 days, and in two cases to 100 days [76]. Normally, the brain is protected from ROS formation by the high concentration of antioxidants such as ascorbic acid, thus preventing the key oxidation of Fe(II) to Fe(III) with consequent QM formation; however, ionizing radiation facilitates this step, thereby allowing expression of the cytotoxic effect in the brain [81]. Finally, mice injected with B16F10 cells (mouse melanoma) in the presence of LNCs loaded with **4** or **27** exhibited significantly extended survival rates, that were explained by the inactivation, by complexes, of pluripotent stem cells [81].

Studies to enhance the effectiveness of these systems are continuing, and some are currently undergoing clinical trials.

6.4 Cytotoxicity of Iron Complexes Compared to those of other Group 8 Metals

Comparison of the biological effects of Group 8 (Fe, Ru, Os) metal complexes derived from tamoxifen reveals the greater cytotoxicity of iron complexes relative to those of Ru and Os. This is particularly true for their mono and diphenolic complexes (IC_{50} values of the diphenolic complexes of Fe, Os and Ru: 0.5, 27, 29 µM, respectively) [20]. One can readily envisage that the quinone methides play a role in this difference since it has been shown that, in the case of osmium, enzymatic oxidation stops at the carbenium ion stage whose reactivity is not comparable to that of a quinone methide [45], and that inhibition of TrxR *in vitro* by the QMs of osmium is less than that induced by iron complexes [44,45]. Moreover, one must also take into consideration the redox potentials of the three metallocenes (+0.4 V for ferrocene, +0.6 V for osmocene, +0.8 V for ruthenocene) and note that, according to Kovacic, only the metallocenes having a redox potential between +0.4 and -0.4 V display anticancer activity since this range of potential is favorable for *in vivo* electron transfer and redox cycling [82].

Only iron complexes meet this criterion; on the other hand, osmium derivatives have shown their usefulness in allowing the study of the localization of complexes within the cell [51].

7 Conclusions and Perspectives

Of all the complexes of ferrocene with antitumoral potential [83–88], the ferrocifens and ferrociphenols have a privileged position owing to their strong anti-proliferative properties against a wide panel of cancer cells. They also benefit from the fact that their mechanism of action is now becoming well understood. Thus, access to their first metabolite, which is key to a succession of biologically significant events, has been closely studied and has revealed a novel chemistry. It has been shown that, starting from a metal-containing tamoxifen-like precursor, chemical or enzymatic oxidation gives rise to a new functional group, namely an organometallic quinone methide (OM-QM), that exhibits previously unknown behaviour.

The initial role of the ferrocenyl redox antenna, the metallocene moiety stabilizing an Fe(II) unit sandwiched between cyclopentadienide rings, is to be converted into its oxidized Fe(III) form as the ferricenium ion, whereupon electronic delocalization is possible along the (*E*)-ferrocenyl-ene-*p*-phenol framework. Subsequent deprotonation, a second oxidation to generate a ferrocenyl-stabilized carbocation, and a final deprotonation, allows the iron to regain its initial +2 state; the resulting quinone methide is a new bioorganometallic entity that behaves as a selective electrophile. The redox potential of the iron is particularly suitable for biology [89,90], such that on cancer cells the ferrocifens initially produce Reactive Oxygen Species (ROS) that unlock the above-mentioned sequence of reactions. Moreover, the ability of the OM-QM to undergo nucleophilic addition of cellular thiols or selenols, that normally control oxidative stress, plays a crucial role in its bioactivity.

In the presence of an antioxidant the sequence no longer operates. Indeed, one OM-QM acts as an *antiproliferative amplifier of oxidative stress* that we have designated as the kronatropic effect (see Section 5) [69]. The chemical and biological reactivity of the OM-QMs is highly dependent on the identity of the substituents, giving these very versatile scaffolds a wide range of options according to their microenvironment. If ferrocene is the redox metallocene best suited to biology, osmocene is an alternative tool particularly suitable as a tracer for cell imaging. For example, it was possible to characterize the presence of

hydroxyosmocifen **48** on MDA-MB-231 cells at the level of the endoplasmic reticulum in the initial phase of the process [51].

Besides apoptosis, these systems can also operate via senescence, offering additional options especially for the ferrociphenols. A number of biological targets, such as thioredoxin reductase, cathepsin B, the estrogen receptor, and the AP1 pathway, provide access to inhibition activities in stem cells. Antiproliferative effects and activity on suppression of metastasis have been used so far as principal selection criteria for anticancer-drugs [75], but this list is not yet exhaustive. For example, Berger has postulated the role of certain metallodrugs in immunogenic cell death [91]. The ferrocifens appear to be good potential candidates to activate this pathway, but this is still in the process of verification. It is already clear that the potential for multisite activity shown by these compounds is of significance in preventing and/or circumventing the phenomena of resistance. Repurposing of drugs is an emerging area in the field of medicine. There is currently much attention given towards repositioning earlier drugs for new therapy. In the ferrocifen family, bactericidal and fungicidal activities have been identified [92], while ferrocenyl chalcone difluroborates inhibited HIV-integrase [93].

Other derivatives of ferrocene scattered throughout the literature have also shown themselves to be particularly active, as for example when a ferrocenyl is adjacent to a naphthyl-dipeptide group [94], or with certain chiral ferrocenyl-nucleosides [95], iron(II) cyclopentadienyl benzonitrile [96], and multi-iron complexes with vinyl-iminium ligands [97], while ferroquin has been remarkably active against malaria [98,99]. Other organometallic series such as those bearing metallacycles also show promise. Examples include certain chiral cyclometalated Pt structures that inhibit cathepsin B [100], highly cytotoxic derivates of Ru that induce a pathway via endoplasmic reticulum stress [101], cyclometalated complexes of Au(III) [102], and cyclic tamoxifens of Mo [103]. Many of these compounds are still at the preliminary stage but look likely to lead to further development. This widening of the field is welcome as it provides a new impetus towards the resolution of some socially very important issues.

All of our compounds are lipophilic and require suitable formulations for administration *in vivo*. This has already been done using lipid nanocapsules [67,77,79,80]. However, the currently exponential growth in drug delivery options suggests that here also considerable further progress is likely to be made in the near future for example with

cyclodextrins [33,104]. All these developments need to be pursued jointly to achieve the benefits of cross-fertilization. In light of the feasibility of the new approaches demonstrated here, it appears that this may lead to a new bioorthogonal chemistry [105]. With the confidence of its recent achievements, and the level of interest it attracts, bioorganometallic chemistry has clearly moved beyond the diffidence of its early days and is emerging more and more fully as an innovative field with the promise of still much more to come.

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