Synthesis, Biochemical Properties and Molecular Modelling Studies of Organometallic Specific Estrogen Receptor Modulators (SERMs), the Ferrocifens and Hydroxyferrocifens: Evidence for an Antiproliferative Effect of Hydroxyferrocifens on both Hormone-Dependent and Hormone-Independent Breast Cancer Cell Lines

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Abstract: A series of ferrocene derivatives based upon the structure of the antiestrogenic drug tamoxifen or of its active metabolite hydroxytamoxifen has been prepared and named by analogy ferrocifens and hydroxyferrocifens. This series includes 1-[4-($O(CH_2)_nNMe_2$)-phenyl]-1-phenyl-2-ferrocenyl-but-1-ene and 1-[4-($-O(CH_2)_nNMe_2$)phenyl]-1-(4-hydroxyphenyl)-2-ferrocenyl-but-1-ene, with $n=2$, 3, 5 and 8, and 1-[4-($-O(CH_2)_2NMe_2$)phenyl]-1-(4-hydroxyphenyl)-2-ferrocenylethene. Most of these molecules have been synthesised by McMurry cross-coupling of the appropriate ketones, except for the ethene complexes, which were prepared by a four-step reaction sequence starting from the ferrocenylacetic acid. All these compounds were obtained as mixtures of *Z* and *E* isomers. The isomers were separated in the cases of the ferrocenyl derivatives of tamoxifen and hydroxytamoxifen ($n=2$). No isomerisation of the *Z* and *E* isomers occurred in DMSO after one day, while a 50:50 mixture of the isomers was obtained within one hour in chloroform. The X-ray structure of (*E*)-1-[4-($-O(CH_2)_2NMe_2$)phenyl]-1-(4-hydroxyphenyl)-2-ferrocenyl-but-1-ene has been determined. The relative binding affinity (RBA) values of the hydroxyferrocifens for the estrogen receptor alpha (ERα) was good to moderate, with values decreasing progressively with the length of the basic chain. The RBA values found for the estrogen receptor beta (ERβ) are equal to or slightly less than those found for the alpha form. The lipophilicity of the hydroxyferrocifens are superior to the values found for estradiol and increase with lengthening of the chain. The antiproliferative effects of the four hydroxyferrocifens with $n=2$, 3, 5 and 8 were studied on four breast cancer cell lines (MCF7, MDA-MB231, RTx6 and TD5) possessing different levels of ERα. On MCF7 cells containing high levels of ERα, hydroxyferrocifens behave as antiestrogens. At a molarity of 1 μM the effect is close to that of hydroxytamoxifen (used for reference) when $n=2$ or 5, more marked when $n=3$, and weaker when $n=8$. Ferrocene alone has no effect. For the MDA-MB231 cells, classed as a hormone-independent breast cancer cell line, on the other hand, the hydroxyferrocifens show remarkable antiproliferative behaviour while the hydroxytamoxifen is completely inactive. Hydroxyferrocifens therefore show the unique property of being active both on hormone-dependent and on hormone-independent breast cancer cell lines. The molecular modelling study provides some clues for understanding of the antagonist effect of these molecules, while an additional cytotoxic effect due to the vectorised ferrocenyl unit is revealed in some occasions.

Keywords: antitumor agents · bioorganometallic chemistry · breast cancer · iron · tamoxifen

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Supporting information for this article is available on the WWW under http://www.chemeurj.org or from the author, including Table S2 (fractional coordinates), Table S3 (interatomic distances), Table S4 (bond angles) and a listing of structure factors for (*E*)-**3a**.

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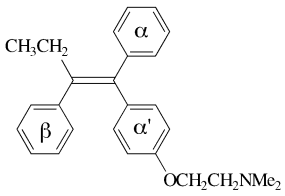
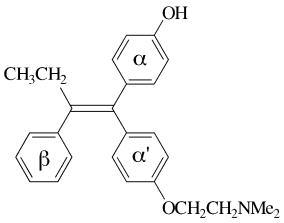
Introduction

Tamoxifen is currently the most widely used antiestrogen in adjuvant therapy of hormone-dependent breast cancers. Its active metabolite is 4-hydroxy-tamoxifen.[1–3]



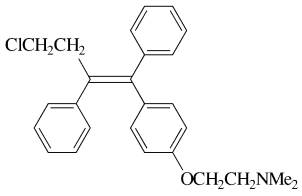
(Z)-Tamoxifen



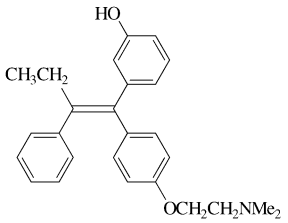
(Z)-HO-Tamoxifen

Since tamoxifen is active only against tumours that are estrogen receptor positive (ER+), and frequently gives rise to resistance after prolonged use, the search for related but different agents has intensified considerably over the last few years.[4–8]

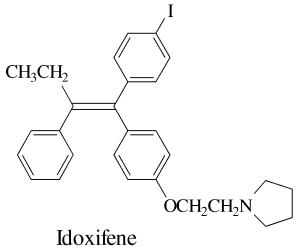
Attention quickly centred on variations on the structure of the triphenylethylene skeleton, resulting in new specific estrogen receptor modulators (SERMs) that include toremifene, droloxifene and idoxifene[7, 9].



Toremifene



Droloxifene



Idoxifene

Derivatives of 2-phenylindoles have also been developed, giving zindoxifene, and also from aryl-benzo[b]thiophenes, which provide access to raloxifene and thence to steroidal compounds modified by the addition of long carbon chains (of around nine carbon atoms), these last compounds behaving as pure antiestrogens.[3, 10] It should be noted that all known SERMs bear a chain of varied length but always with p or π electron sites (e.g., $-NR_2$, −S−R, −SO−R, $-SO_2R$ etc.).

It recently became possible to rationalise this observation when the X-ray crystal structure of the ligand-binding domain (LBD) of estrogen receptor alpha was elucidated.[11–15] This study showed that the bioligand can bind as an antagonist, creating a new pocket in a flexible area of the protein and allowing accommodation of the basic chain. The result of these conformational effects is to modify the position of helix 12 of the receptor, preventing it from interacting with certain effectors present in the target cell and therefore from carrying out its function as an activator of specific genes.

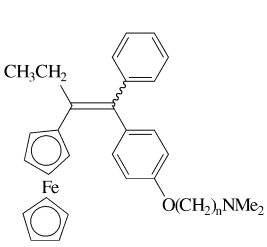
Tamoxifen acts in vivo as a particularly well tolerated cytostatic agent. It should be noted that the molecule exists both in *Z* and in *E* configurations, of which the *Z* isomer has been shown to be the most strongly antiestrogenic, but because the molecule isomerizes in solution, it is the mixture that is administered. This may explain a certain duality in the effects of tamoxifen, but it has not proved a barrier to its success.

The hydroxy group is essential in the active metabolite of this molecule, since it confers an increased level of affinity for the estrogen receptor. As is well known, this receptor plays a key role in the proliferation of hormone-dependent tumours and is one of the most important target molecules for tamoxifen, although other targets do exist.[16]

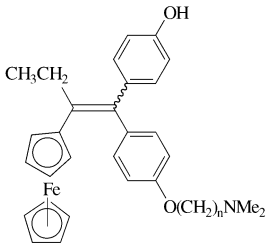
Considerable interest has been generated by coordination compounds of platinum, and so a strategy has been developed based on coupling of platinum, with its known efficacy in chemotherapy and high general cytotoxicity, to various ligands of the estrogen receptor through a nitrogen atom, with the aim of creating antiestrogenic compounds that are able both to target the binding site and to provide increased cytotoxicity.[17–21] Unfortunately the Pt−N coordination bond in these complexes is too weak and it appears that the metal is unable to reach the target because it hydrolyses too quickly and is too bulky in size. This is the limiting factor in the appealing strategy of coupling a transporter group to a coordination complex in order to increase its cytotoxicity.

However, this concept of the antiestrogenic transporter can be revisited from another perspective. Instead of compounds with coordination bonds, which are inherently quite weak, we may envisage the use of species with strong metal–carbon covalent bonds, and here we enter the field of organometallic chemistry. Metallocenes are in fact known to have their own antitumor properties, based on a different mechanism from that of the cisplatin complexes.[22] To our knowledge, however, previous to our own work they have not been incorporated into an antiestrogenic entity. The idea of attempting this, as a means towards the discovery of antiestrogens with improved cytotoxic properties, was appealing.

Ferrocene $(\eta^5\text{-}C_5H_5)_2Fe$ is the archetypal metallocene. One could envisage using it to increase the cytotoxicity of hydroxytamoxifen by attaching this moiety to the key skeleton of diphenylethylene bearing the crucial hydroxy and dimethylamino groups. In fact, a ferrocenyl moiety could present a number of advantages. Ferrocene is inherently more aromatic than benzene, so it is likely that replacement of the β phenyl nucleus of 4-hydroxy-tamoxifen would be well justified; it is chemically fairly stable in various non-oxidizing media and has been reported to have antitumor activity due to metabolic formation of ferricinium ions in situ.[23] Other metallocenes with metals such as Ti,[24] V, Mo, Re,[25] Co and Ru may also be envisaged. To test the validity of the concept, we synthesised derivatives of ferrocene following the base structure of tamoxifen, in which the aromatic β ring is replaced by a ferrocenyl group and the dimethylamino side chain is of variable length. By analogy these derivatives were named ferrocifens and hydroxyferrocifens.



(*Z* + *E*)-Ferrocifen



(*Z* + *E*)-HO-Ferrocifens (*n* = 2, 3, 5 ,8)

Preliminary studies revealed that the length of the chain appeared to have a considerable effect on the biological properties of the complexes.[26, 27] Here we present the synthesis, molecular modelling studies and biological properties of a series of ferrocifens and hydroxyferrocifens.

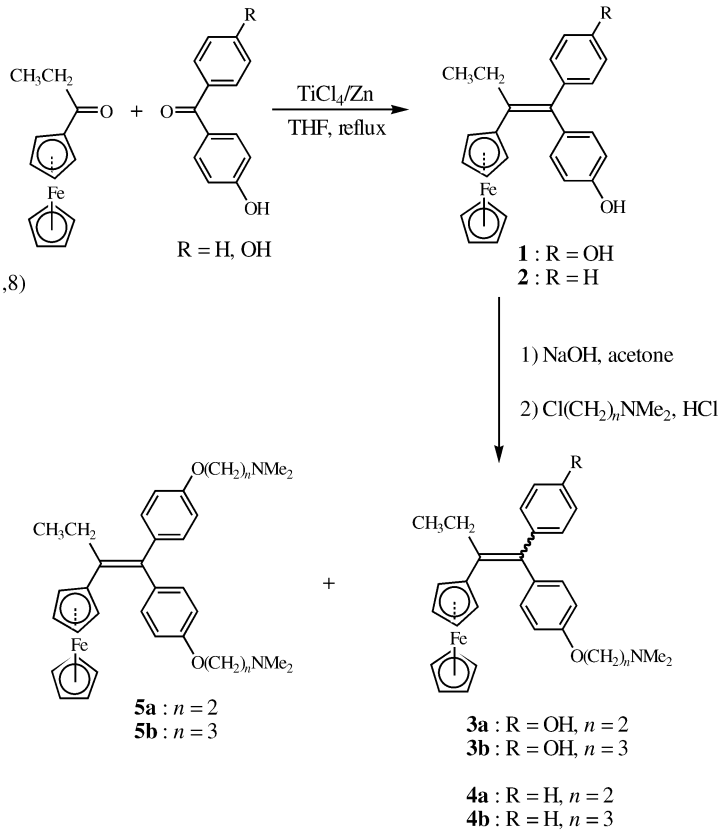
Results and Discussion

Synthesis of the products: The first hydroxyferrocifens were prepared in our laboratory by starting from ethyl ferrocenylacetate and using a long, painstaking and impractical method.[28] After testing several synthetic routes, we found that a route based on McMurry coupling (Zn, $TiCl_4$) was the most convenient, since it is shorter when starting from metallocene ketones.[29] It is easy to perform and gives good yields of mixtures of *Z* and *E* isomers. This method has since been extended to all the complexes described here. Two synthetic strategies were adopted, depending on the commercial availability of the particular reagent $X(CH_2)_nNMe_2$. In cases in which the reagent is available (i.e., for $n = 2$ and 3), the synthesis begins with a coupling reaction between propionylferrocene, obtained by alkylation of the ferrocene by means of a Friedel–Crafts reaction, and hydroxybenzophenone or dihydroxybenzophenone (Scheme 1).

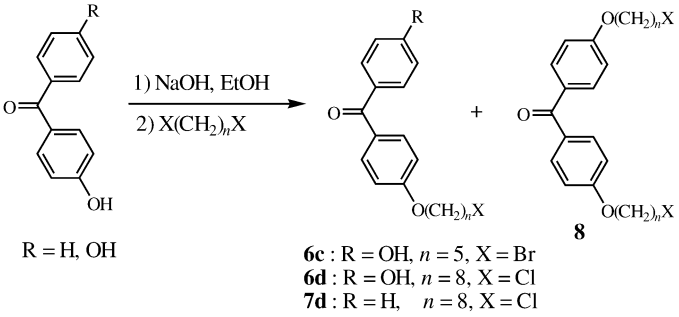
After the propionylferrocene/dihydroxybenzophenone mixture has been stirred in the presence of the McMurry reagent, the complex **1** resulting from the cross coupling is obtained as the major product with a yield of 52%.[26] Coupling with hydroxybenzophenone gives **2** as a mixture of two isomers (*Z* and *E*).[29] Heating of these two compounds with sodium hydroxide in acetone, followed by addition of the chloroalkyldimethylamine derivative, gives the hydroxyferrocifens **3a** and **3b** and ferrocifens **4a** and **4b** in yields ranging between 26% and 66%. Alkylation of **1** similarly gives the dialkylation compounds **5a** and **5b**.

In cases in which the reagent giving access to the amine chain $X(CH_2)_nNMe_2$ is not commercially available (i.e., for $n = 5$ and 8), it is necessary to start with alkylation of the mono- or dihydroxybenzophenone by an α,ω-dihaloalkane (Scheme 2). This gives the monoalkylated derivatives **6c**, **6d** and **7d**, as well as the dialkylated derivative **8** (in the case of dihydroxybenzophenone).

The obtained ketones are then coupled with propionylferrocene in the presence of the McMurry reagent to give the halogenated compounds **9c**, **9d**, **10c** and **10d** (Scheme 3). In the final step, these derivatives are allowed to react with



Scheme 1. Synthesis of ferrocifens and hydroxyferrocifens with $n = 2$ or 3 by McMurry cross-coupling.

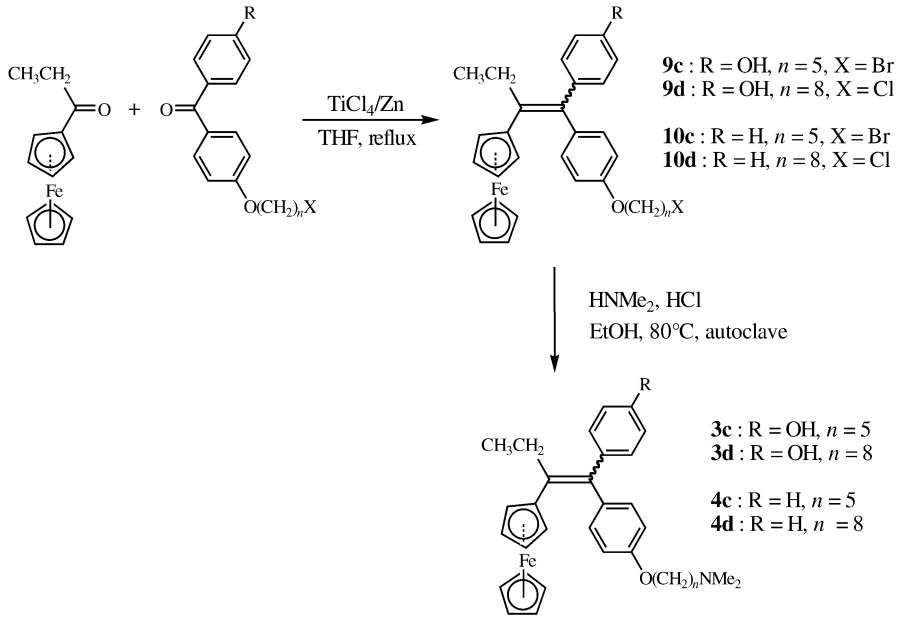


Scheme 2. Synthesis of ketones **6c**, **6d** and **7d**, bearing amino chains $O(CH_2)_nX$ ($n = 5$, 8).

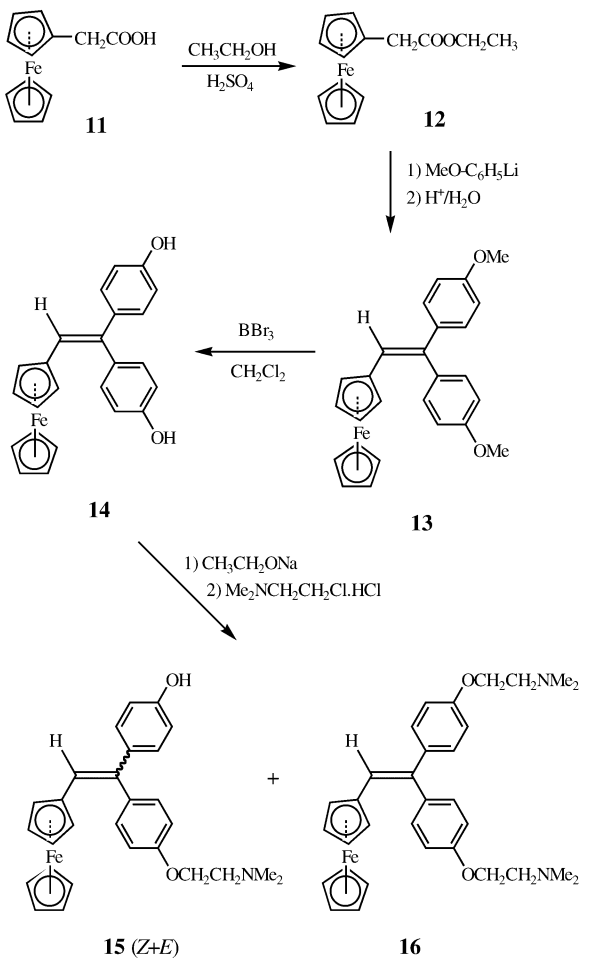
dimethylamine hydrochloride in an autoclave. This gives the hydroxyferrocifens **3c** and **3d** and the ferrocifens **4c** and **4d**.

For complexes **14** and **15**, in which a hydrogen replaces the ethyl group, the synthesis is performed from ferrocenylacetic acid (**11**) by the route shown in Scheme 4.

The acid **11** is first converted into the ester **12**. Treatment of **12** with methoxyphenyllithium gives an alcohol intermediate, which dehydrates to give 1,1-bis(4-methoxyphenyl)-2-ferrocenylethene (**13**). Demethylation of **13**, to give the diphenol **14**, is then induced by treatment with BBr_3 in dichloromethane. The final step is to attach the dimethylamino chain onto one of the two phenolic functions of **14**. Use of sodium ethanolate followed by 2-chloroethyl-dimethylamine hydrochloride gives a mixture of the two isomers (*Z* + *E*) of **15** in 41% yield for this final step. The secondary dialkylated compound **16** is also isolated (30%).



Scheme 3. Synthesis of ferrocifens and hydroxyferrocifens with $n = 5$ or 8 by McMurry cross-coupling.



Scheme 4. Synthesis of hydroxyferrocifen **15** from ferrocenylacetic acid (**11**).

All the ferrocifens and hydroxyferrocifens are obtained as mixtures of Z and E isomers. It was possible to separate the isomers in the cases in which $n = 2$ (complexes **3a** and **4a**). For hydroxyferrocifen **3a**, separation was achieved by fractional crystallisation from an ethyl ether/hexane mixture. In the case of ferrocifen **4a**, the Z and E isomers were separated by plate chromatography after several treatments with a toluene/pyridine (6:1) eluent. Separation of the other isomers ($n = 3$, 5 and 8) by these methods was not possible.

Structural study: Identification of the structures of the Z and E isomers can only be accurately determined by X-ray crystallographic study. After separation of the Z and E isomers, the complexes **3a** were allowed to crystallise from diethyl ether/hexane (5:1) mixtures. Only the E isomer of **3a** gave crystals of sufficient quality to perform an X-ray structural analysis. The crystals were monoclinic and belonged to the $P2_1/c$ space group. The crystallographic data are given in Table 1, and the ORTEP diagram of (*E*)-**3a** is shown in Figure 1. To facilitate comparisons between the structures of ferrocifen **4a** and hydroxyferrocifen **3a** we have also included the ORTEP diagram for (*Z*)-**4a**, the structure of which was published in an earlier paper.[29]

Table 1. Summary of crystallographic data for (*E*)-**3a**.

formula	$C_{30}H_{33}O_2NFe$
Fw	495.4
a [Å]	15.174 (4)
b [Å]	13.259 (4)
c [Å]	14.197 (18)
β [°]	112.17 (1)
V [Å^3]	2645(1)
Z	4
crystal system	monoclinic
space group	$P2_1/c$
$F(000)$	1048
linear absorption coefficient μ [cm^{-1}]	6.56
ρ [g cm^{-3}]	1.24
diffractometer	Enraf–Nonius CAD4
radiation	$Mo_{K\alpha}$ (0.71070 Å)
scan type	$\omega/2\theta$
scan range (°)	$0.9 + 0.34\ \mathrm{tg}\theta$
θLimits (°)	4–50
temperature of measurement	room temperature
octants collected	0,8; 0,18 ; 0,22
no. of data collected	4579
no. of unique data used for refinement	1034 ($I > 3\sigma(I)$)
$R = \Sigma \vert\vert F_o\vert - \vert F_c\vert\vert / \Sigma \vert F_o\vert$	0.053
Rw[a] $= \{\Sigma w(\vert F_o\vert - \vert F_c\vert)^2 / \Sigma w F_o^2\}^{1/2}$	0.057
absorption correction	DIFABS (min = 0.90, max = 1.16)
extinction parameter	none
goodness of fit s	1.17
no. of variables	149
$\Delta\rho$min [e Å^{-3}]	−0.47
$\Delta\rho$max [e Å^{-3}]	0.62

[a] $w = w'[1 - ((\vert\vert F_o\vert - \vert F_c\vert\vert)/6\sigma(F_o))^2]^2$ with $w' = 1/\Sigma r A_r T_r(X)$ with three coefficients 3.79, −0.810 and 2.74 for a Chebyshev Series, for which X is F_c/F_c(max).

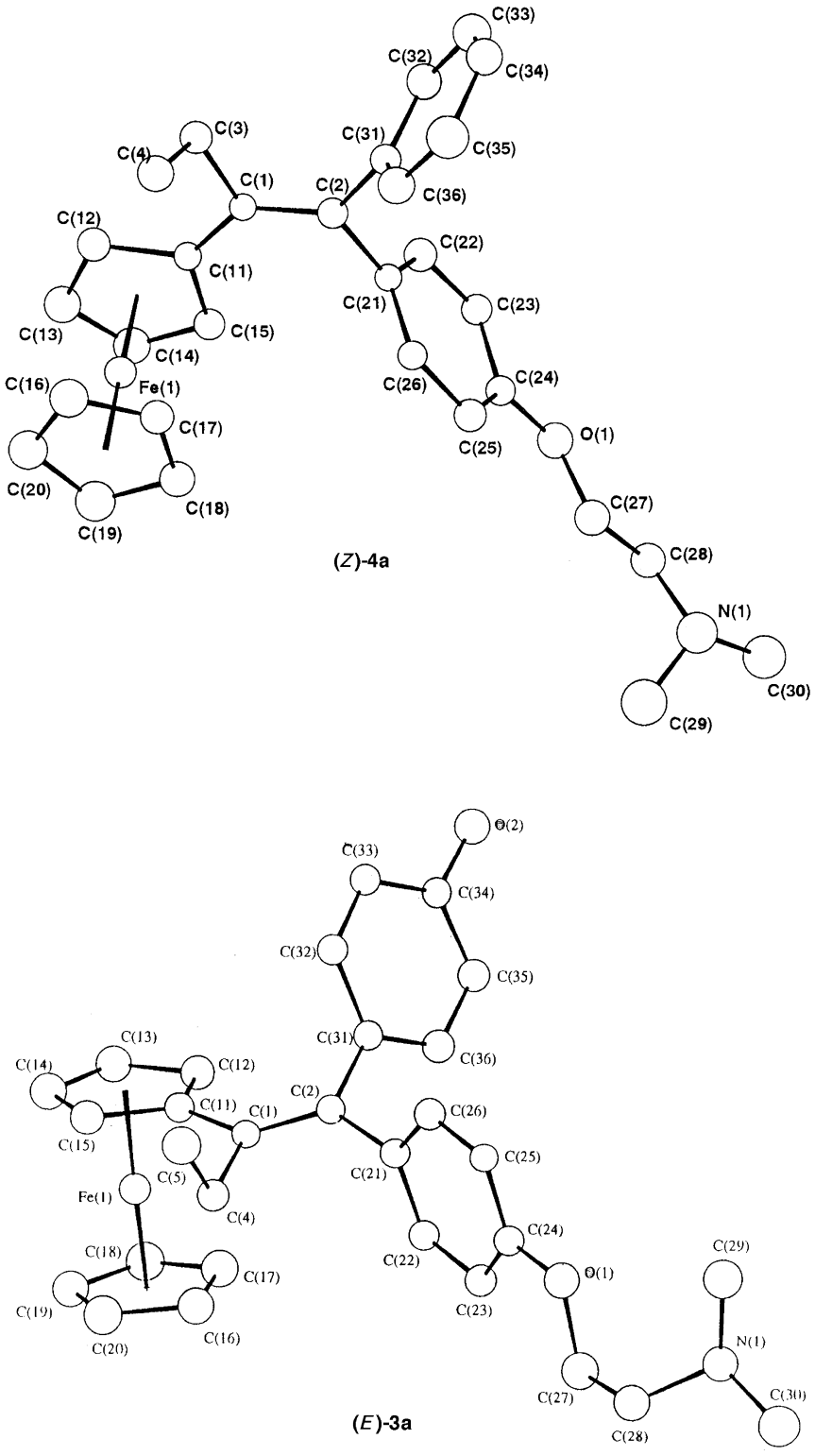
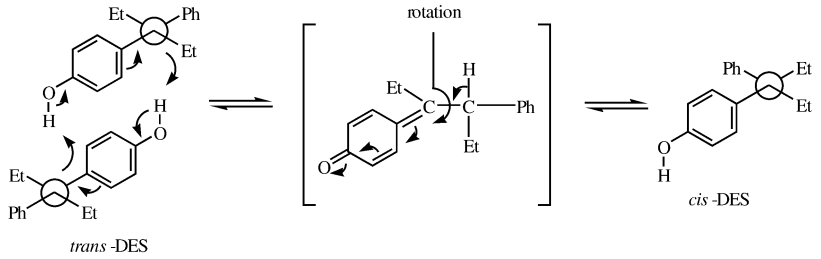


Figure 1. X-ray structures of (*Z*)-**4a** and (*E*)-**3a**.

The structures of (*E*)-**3a** and (*Z*)-**4a** may be compared to those of *cis*- and *trans*-tamoxifen determined by Kilbourn[30] and Precigoux,[31] respectively. The following points may be noted: the interatomic distances in the central double bonds (C(1)=C(2)) are 1.36 Å in (*E*)-**3a** and 1.37 Å in (*Z*)-**4a**, while that in *cis*-tamoxifen (*E*) is 1.33 Å and that in *trans*-tamoxifen (*Z*) is 1.34 Å. The ferrocenyl group appears in its normal conformation, with the iron atom bound symmetrically to the two cyclopentadienyl rings, with an average C–Fe distance of 2.04 Å. A slight deformation is observed at the level of the ethylene skeleton. The angle formed by the double bond and one of the four substituents is of the order of 122–124° for both the *cis*- and the *trans*-tamoxifens. In the case of (*E*)-**3a**, the bond angles have the following values: C(11)-C(1)-C(2): 126.2°, C(1)-C(2)-C(31): 124.6°, C(4)-C(1)-C(2): 119.0°, and C(1)-C(2)-C(21): 120.9°. In the case of (*Z*)-**4a**, the bond angles have the following values: C(11)-C(1)-C(2): 129.0°, C(1)-C(2)-C(31): 125.0°, C(3)-C(1)-C(2): 114.4°, and C(1)-C(2)-C(21): 122.0°. There is thus a widening of the angle at the side at which the ferrocenyl group is located, and a narrowing on the ethyl side. This is probably attributable to the greater bulk of the ferrocenyl group relative to the phenyl. It can also be seen that the four substituents on the double bond do not lie in the same plane, the C(11)-C(1)-C(2)-C(31) atoms forming a dihedral angle of 13° in the case of (*E*)-**3a**, while in (*Z*)-**4a** they form a dihedral angle of only 1.25°. The twist angle formed between the plane of the aromatic ring and the plane of the ethylene skeleton often attracts the attention of researchers. This angle has been found to be of the order of 52° to 55° for the three rings of *cis*-tamoxifen, and similar values have also been found for *trans*-tamoxifen. In the case of (*E*)-**3a**, the C_6H_4–OH ring forms an angle of 55° with the plane of the double bond, in agreement with those of (*Z*)- and (*E*)-tamoxifen. In contrast, the C_6H_4–OCH_2CH_2–NMe_2 ring forms an angle of 74°, about 20° greater than the listed value. The distance between the O(1) and O(2) groups in (*E*)-**3a** is 9.65 Å. Finally, we note that the distance between O(3) and O(17) is 10.9 Å for estradiol[32] and 12.1 Å for diethylstilbestrol (DES).[33] This X-ray analysis shows that the hydroxyferrocifens have the size characteristics of a nanomediator for the estrogen receptor.

Isomerisation of (*Z*)-Fc-hydroxytamoxifen ((*Z*)-3a): Isomerisation leading to interconversion between *Z* and *E* isomers in the stilbene series is a well known phenomenon. This isomerisation causes a serious problem in determination of the exact nature of the more biologically active isomer. In general, the *Z* isomer shows higher affinity for the specific estrogen receptor (ER) than the *E* isomer does. This is the case, for example, with DES. The isomerisation phenomenon has been carefully studied by Winkler et al. for DES[34] and by Robertson and Katzenellenbogen for hydroxytamoxifen.[35] These studies led the authors to propose the isomerisation mechanism shown in Scheme 5.



Scheme 5. Proposed mechanism for the isomerisation of DES.[34]

The isomerisation is considered to be a consequence of the interaction between the central double bond and the hydroxy group of the phenol. This is a bimolecular process that implies a transfer of the phenolic proton onto the double bond. It has also been shown that this isomerisation was catalysed by Lewis acids.[36] Winkler et al., studying the influence of the solvent type on the speed of isomerisation of DES, found that isomerisation became slower in the order benzene > heptane > chloroform > ethyl acetate > pyridine > *n*-butanol > acetone > methanol, corresponding to the increase in the dielectric constant of the solvent. The authors conclude that the isomerisation of DES is favoured by solvents with low dielectric constants.

In our case we studied the isomerisation of (*Z*)-hydroxyferrocifen **3a**. It should be noted that isomerisation occurs only in solution and not in the solid state. Acetone, benzene, chloroform and DMSO were selected as solvents for the study, which was carried out by NMR spectroscopy by the following method: for each solvent studied, a solution of (*Z*)-**3a** was placed in an NMR tube and left at room temperature for 1 h, and formation of the *E* isomer was monitored. The percentage of *E* isomer formed was determined by measuring the intensity of the least shielded doublet of the aromatic protons of each isomer. For example, in [D_6]DMSO the isomer (*Z*)-**3a** shows a doublet at $\delta = 6.97$ ppm quite distinct from the doublet at $\delta = 7.08$ ppm belonging to the (*E*)-**3a** isomer. Table 2 summarises the results obtained.

After 1 h, only chloroform allowed formation of a 50:50 mixture. Isomerisation was rapid, with a 30% concentration of the *E* isomer found after only 15 min. Equilibrium was reached after one hour. At the end of one day, a 50% share of isomer *E* was achieved in acetone, but this share remained at 0% in benzene and DMSO.

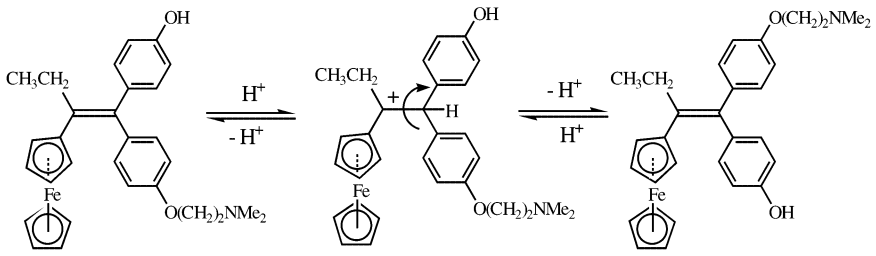
Table 2. NMR study of the influence of the solvent on the speed of isomerisation of (*Z*)-**3a**.

	Solvent			
(*Z*)-**3a**/(*E*)-**3a** (%)	[D_6]Benzene	$CDCl_3$	[D_6]Acetone	[D_6]DMSO
after one hour	100:0	50:50	100:0	100:0
after one day	100:0	50:50	30:70	100:0

The correlation between speed of isomerisation and the dielectric constant of the solvent, as observed by Winkler for DES, is thus not borne out in the case of ferrocifen (*Z*)-**3a**. The clearest example is in the case of benzene, in which no isomerisation of the (*Z*)-**3a** complex was observed. Here we must make recourse to a feature specific to organometallic chemistry. It is in fact known that organometallic complexes adjacent to a double bond favour stabilization of α-carbenium ions by protonation of the double bond. The pK_a values of chloroform, acetone, DMSO and benzene are 15, 20, 31 and 37, respectively. Chloroform is thus the most acidic of the solvents, followed closely by acetone. The speed of isomerisation observed for (*Z*)-**3a** (chloroform > acetone > DMSO ≥ benzene) may therefore be correlated with the acidity of the solvent. In the present case, the speed of isomerisation of the complex seems to be controlled by the ease of formation of the carbenium ion, following the mechanism shown in Scheme 6.

Biochemical Studies

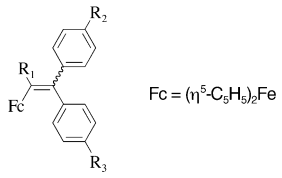
Measurement of the relative binding affinities (RBAs) of the complexes for the estrogen receptors: The RBA values of all the complexes were measured for the alpha form of the estrogen receptor (ERα). For the most representative complexes of the series these RBA values were also measured for the recently reported[37, 38] beta form (ERβ).



Scheme 6. Proposed mechanism for the isomerisation of hydroxyferrocifen **3a**.

Study of some Z *and* E *isomers of ferrocifens: Influence of the solvent, of incubation time and temperature (Table 3)*: In the ferrocifen and hydroxyferrocifen series the *Z* and *E* isomers were separated for $n=2$ (**3a** and **4a**), and their RBA values for ERα were determined at two temperatures (0 °C and 25 °C) starting from stock solutions (1 mM) prepared in ethanol or DMSO (Table 3). These results were compared to those obtained for (*Z*)-hydroxytamoxifen and the mixture of the two isomers. The results show that in the hydroxyferrocifen series the RBA values obtained from stock solutions prepared with DMSO (final percentage DMSO = 5 %) are between two and five times higher than those obtained with ethanol solutions. This is not observed for hydroxytamoxifen. Complex **3a** shows good recognition by the estrogen receptor (RBA = 14.6 % for the (*Z* + *E*) mixture). As in hydroxytamoxifen, the (*Z*)-**3a** isomer (RBA = 40 %) gives better recognition than the (*E*)-**3a** isomer (RBA = 12 %). However these values are lower than those found for hydroxytamoxifen, probably because of increased steric hindrance of the ferrocene relative to that of a benzene ring. As in the case of hydroxytamoxifen, the RBA values of **3a** decrease when the incubation temperature is raised from 0 °C to 25 °C. Unsurprisingly, complexes without a hydroxy group—(*Z*)-**4a** and (*E*)-**4a**—have very low RBA values (less than 1 %).

Table 4. Relative binding affinities (RBAs) of some ferrocifens for ERα.



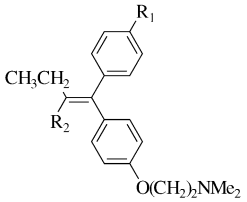
Compound	R^1	R^2	R^3	RBA(%) for ERα ethanol (0 °C, 3 h30)	DMSO (0 °C, 3 h30)
5a	Et	$O(CH_2)_2NMe_2$	$O(CH_2)_2NMe_2$	0.3	0.6
9c	Et	OH	$O(CH_2)_5Br$	0.4	0.5
9d	Et	OH	$O(CH_2)_8Cl$	0.01	0.01
14	H	OH	OH	6[a]	20[a]
15	H	OH	$O(CH_2)_2NMe_2$	n.d.	17[b]
17[c]	Et	OH	OH	5.4	9.4[b]

[a] Means of three experiments. [b] Means of two experiments. [c] Prepared as described in reference [26]. n.d. = not determined.

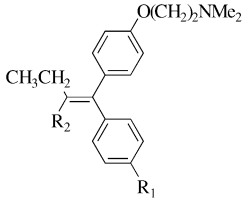
Influence of various R^1, R^2 and R^3 substituents on diphenyl ethylene ferrocenes (Table 4): Replacement of the ethyl chain with a hydrogen (compounds **14** and **15**) causes a slight increase in the RBA value for complexes with a dimethylamino side chain (RBA = 14.6 for **3a** and 17 for **15**). The increase is much more pronounced in the case of the diphenols (RBA = 9.4 for **17** and 20 for **14**). Replacement of the side chain with a hydroxy group leads either to a slight increase or a decrease in RBA (17 and 20 % for **15** and **14**, 9.4 and 14.6 % for **17** and **3a**). Lengthening of the chain and substitution of a bromine or a chlorine for the dimethylamino group of the same chain leads to almost complete loss of affinity for the receptor (RBA from 0.5 and 0.01 for **9c** and **9d**). Finally, complex **5a**, with no hydroxy group, has a very low RBA value.

Influence of chain length in the ferrocifen series (Table 5): The RBA values of the ferrocifens (**3a**–**3d**) possessing chains of varying lengths ($n=2$, 3, 5 and 8) were determined for the mixtures of the two isomers ($Z + E$) on both the alpha and beta forms of the estrogen receptor (ERα and ERβ). Complexes **3a** and **3b**, with the shortest chains, have fairly high RBA values (greater than or equal to 10 %) and thus show good recognition by ERα. As the chain is lengthened

Table 3. Relative binding affinity (RBA) for ERα and log Po/w values of hydroxytamoxifen (HO-TAM) and of ferrocifens and hydroxyferrocifens. Influence of the solvent (alcohol or DMSO) used to prepare the stock solution of the compounds and of the incubation temperature (0 °C or 25 °C).



Z isomer

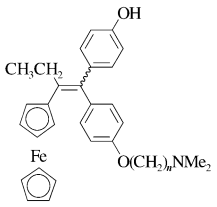


E isomer

Compound	R^1	R^2	RBA(%) on ERα ethanol (0 °C, 3 h30)	DMSO (0 °C, 3 h30)	DMSO (25 °C, 3 h30)	log Po/w[d]
(*Z* + *E*)-HO-TAM	OH	Ph	38.5[b]	38.5[b]	n.d.	–
(*Z*)-HO-TAM	OH	Ph	92	107	51	3.2
(*Z* + *E*)-**3a**	OH	Fc[a]	7.6	14.6[b]	n.d.	–
(*Z*)-**3a**	OH	Fc[a]	7.2[c]	40[c]	10.5	3.8
(*E*)-**3a**	OH	Fc[a]	6.9	12[c]	3.2	3.9
(*Z*)-**4a**	H	Fc[a]	n.d.	0.9	0	5.2
(*E*)-**4a**	H	Fc[a]	n.d.	0.014	0	5.0

[a] Fc = [(η5-C5H5)2Fe]. [b] Mean of two experiments. [c] Mean of three experiments. [d] Measured by HPLC as described in reference [39]. n.d. = not determined.

Table 5. Influence of the length of the chain on the RBA values for ERα and ERβ and on the log Po/w values.[a]



Compound	n	RBA (%) ERα (0 °C, 3 h 30)	ERα (25 °C, 3 h 30)	ERβ (0 °C, 3 h 30)	log Po/w
estradiol		100[b]	100[b]	100[b]	3.5
HO-TAM		38.5	n.d.	24	$Z=3.2$ $E=3.4$
3a	2	14.6	n.d.	10[c]	$Z=3.8$ $E=3.9$
3b	3	11.5[d]	9.2[d]	12[d]	$Z=4.3$ $E=4.5$
3c	5	5[c]	4.6[c]	6[d]	$Z=4.7$ $E=4.8$
3d	8	2.3[c]	2.4	n.d.	$Z=6.0$ $E=6.3$

[a] All the tested compounds are mixture of both isomers ($Z + E$). Measurements were performed with stock solutions in DMSO. [b] Value by definition. [c] Mean of two experiments. [d] Mean of three experiments.

($n=5$ and $n=8$) the RBA values decrease progressively. For the beta form of the receptor the values found are equal to or slightly less than those found for the alpha form.

Determination of log Po/w values: The lipophilicities (log Po/w) of most of these complexes were determined by HPLC by the method described by Pomper et al.[39] Even in those cases in which the two isomers were not separated, it was possible to obtain a log Po/w value for each of the isomers, since they give two distinct peaks in analytical HPLC. The attribution of the Z and E isomers can then be made by analogy with the retention time found for (Z)-**3a**, the crystal structure of which has been determined (see above), and the retention time of the two isomers in the ($Z+E$)-**3a** mixture. Analogously for the whole series, the lowest log Po/w values are attributed to isomer Z, the reference value being that of estradiol (log Po/w = 3.5). It is interesting to note that all the organometallic complexes have log Po/w values higher than those of estradiol, while those of the two isomers of hydroxytamoxifen are lower. This implies that the ferrocifens can enter the cell more easily than hydroxytamoxifen. The values increase progressively as the chain is lengthened. As expected, elimination of the hydroxy group in the complexes produces a noticeable increase in lipophilicity.

Study of the antiproliferative effect of the complexes on hormone-dependent and hormone-independent breast cancer cell lines: The antiproliferative effects of the four hydroxyferrocifens (**3a**–**3d**) and of ferrocene (Fc) were studied on four breast cancer cell lines (MCF7, MDA-MB231, RTx6 and TD5) but with different levels of ERα: a high level for MCF7, moderate for RTx6, low for TD5 and zero for MDA-MB231. RTx6 and TD5 are MCF7 mutants selected for tamoxifen resistance. The results obtained after five days of culture, expressed as percentages of DNA compared to their own controls, that is with or without E_2 are shown in Figure 2, Figure 3, Figure 4 and Figure 5. Thus, a value less than 100% shows an antiproliferative effect, a value of exactly 100% no effect, and a value higher than 100% represents a proliferative effect. On MCF-7 cells, two incubation molarities (1 μM and 0.1 μM) were tested in the absence and in the presence of 10 nM estradiol (Figure 2). As expected, the hydroxytamoxifen used for reference has a strong antiproliferative effect. Ferrocifens **3a**, **3b** and **3c** all have a significant dose-dependent antiproliferative effect. At a molarity of 1 μM, the effects observed with **3a** ($n=2$) and **3c** ($n=5$) are very close to that of hydroxytamoxifen, but are more marked with **3b** ($n=3$). When the chain is lengthened (**3d**; $n=8$) the antiproliferative effect is weakened. Ferrocene alone has no effect. This lack of effect cannot be attributed to a failure of the compound to enter the cell, since the log Po/w value found for ferrocene is 3.3, very close to that of estradiol, so the presence of a transporting group must be required. At this stage it appears that the ferrocifens behave as antiestrogens. This hypothesis can be tested simply by adding estradiol and observing whether or not the effect is reversed. This does indeed occur at an incubation molarity of 0.1 μM, but the effect is much less at a molarity of 1 μM, especially with complexes **3b** and **3c**. This may indicate a dual effect for these complexes that is both antihormonal and cytotoxic. The effect of the products was next tested on the ERα-negative cell line MDA-MB231 (Figure 3). In this case hydroxytamoxifen has no effect, as expected since the observed antiproliferative effect is known to be essentially an antihormonal effect mediated by the presence of ERα. In contrast, the ferrocifens, except for **3d**, show a very marked antiproliferative effect, while ferrocene has no effect. This result highlights the ferrocifens' novel property of showing activity both on hormone-dependent and

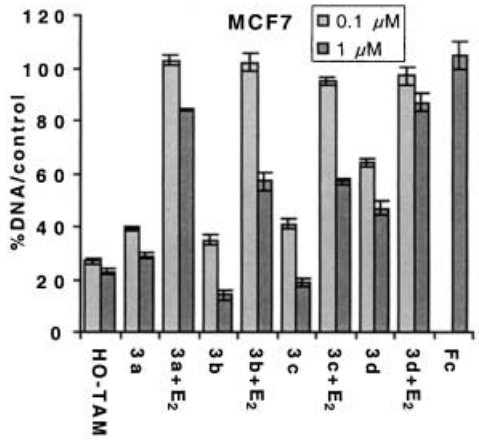


Figure 2. Effects of 0.1 μM and of 1 μM hydroxytamoxifen (HO-TAM), hydroxyferrocifens (**3a**, $n=2$; **3b**, $n=3$; **3c**, $n=5$; **3d**, $n=8$) and ferrocene (Fc) on the proliferation of MCF7 cells (cancer cells with a high level of ERα) after five days of culture in the absence or presence of E_2 (10 nM). Experiment in quadruplicate ± limits of confidence ($P=0.1$, $t=2.35$; 100% without E_2 : 26.6 ± 2.1 μg DNA, with E_2: 30.0 ± 1.7 μg DNA).

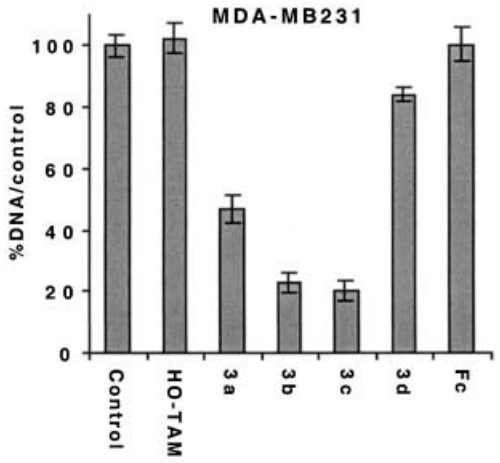


Figure 3. Effect of 1 μM hydroxytamoxifen (HO-TAM), hydroxyferrocifens (**3a**, $n=2$; **3b**, $n=3$; **3c**, $n=5$; **3d**, $n=8$) and ferrocene (**Fc**) on the proliferation of MDA-MB231 cells (breast cancer cells ERα-negative) after five days of culture. Experiment in quadruplicate ± limits of confidence ($P=0.1$, $t=2.35$; 100%: 10.3 ± 0.2 μg DNA).

on hormone-independent cell lines. This difference in behaviour between hydroxytamoxifen and the ferrocifens is also seen with the TD5 cell line (Figure 4), which has a low level of ERα. In this case, in fact, a distinctly more marked antiproliferative effect is found for the three ferrocifens **3a**, **3b**

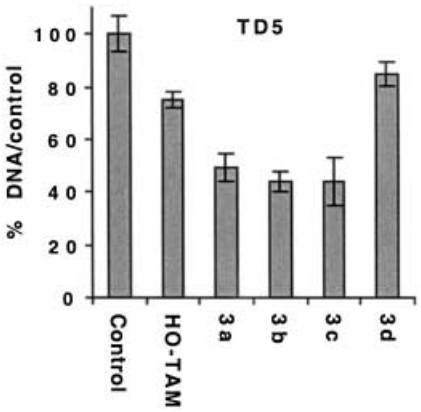


Figure 4. Effect of 1 μM hydroxytamoxifen (HO-TAM), hydroxyferrocifens (**3a**, $n=2$; **3b**, $n=3$; **3c**, $n=5$; **3d**, $n=8$) on the proliferation of TD5 cells (MCF7 mutant cells with low level of ERα) after five days of culture. Experiment in quadruplicate ± limits of confidence ($P=0.1$, $t=2.35$; 100%: 10.6 μg ± 1.0 μg DNA).

and **3c** than for hydroxytamoxifen (50–55% for the ferrocifens, 25% for hydroxytamoxifen). In the RTx6 cell line (Figure 5), with a mid-range level of ERα, hydroxytamoxifen and the hydroxyferrocifens **3a**, **3b** and **3c** show a weak effect (inhibition around 20%) at an incubation concentration of 1 μM, but it is surprising to note that after addition of a small quantity of estradiol (10 nM) the antiproliferative effect observed in the presence of **3b** and **3c** is clearly superior, with an inhibition level reaching 55 or 60% in these cases. It appears that in the presence of estradiol, which has a high affinity for ERα, the antihormonal effect of the ferrocifens is masked, and their cytotoxic action is therefore favoured instead.

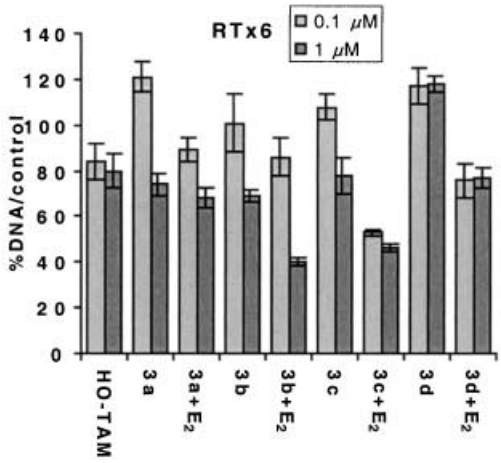


Figure 5. Effect of 0.1 μM and 1 μM hydroxytamoxifen (HO-TAM) and hydroxyferrocifens (**3a**, $n=2$; **3b**, $n=3$; **3c**, $n=5$; **3d**, $n=8$) on the proliferation of RTx6 cells (MCF7 mutant cells with medium level of ERα) after five days of culture in the absence or presence of E_2 (10 nM). Experiment in quadruplicate ± limits of confidence ($P=0.1$, $t=2.35$; 100% without E_2: 10.4 ± 0.8 μg of DNA, with E_2: 14.5 ± 0.4 μg of DNA).

Discussion and Future Perspectives

The results obtained on the antiproliferative effects of the hydroxyferrocifens, particularly with products possessing three- or five-carbon chains, which have more marked effects, may be summarised as follows. With MCF-7 cells, considered the archetypal hormone-dependent breast cancer cell line (ER+), the effects of the hydroxyferrocifens are very similar to those of hydroxytamoxifen, with the two categories of products showing antiproliferative effects of the same order (a little better for hydroxytamoxifen at a concentration of 0.1 μM and better for hydroxyferrocifens ($n=3$, 5) at 1 μM. For the MDA-MB231 cells, classed as a hormone-independent breast cancer cell line (ER−), on the other hand, the effects are spectacularly different. The organometallic complexes show remarkable antiproliferative behaviour, while the hydroxytamoxifen compound is completely inactive.

Before attempts to explain this important result, it may be useful to mention some recent advances in fundamental endocrinology, concerning the discovery of a second estrogen receptor found in various sites, including the prostate and ovaries and in breast cancer.[40] This means that we now need to deal with two estrogen receptors: ERα and the recently discovered ERβ. With this in mind, the MCF7 cell-line, which was previously classed as ER(+), must now be assumed to contain ERα and, to a lesser degree, ERβ. Meanwhile, the MDA-MB231 cell line, originally classified as ER(−), in fact contains ERβ, but not ERα.[41] It has been suggested that tumours that are unaffected by tamoxifen could be attacked via the newly discovered ERβ, although the role of this receptor remains to be elucidated.[2]

Also recently, X-ray crystal structures of the ligand-binding domains (LBDs) of these receptors bound to various bioligands have become available.[11–15] In addition, two mechanistic routes for activation at the level of the DNA, those of the estrogen response element (ERE) and of activator protein 1 (AP1), have recently been identified.[42]

All these effects must now be taken into account, despite the fact that they add a greater degree of complexity to the problem. They do at least give us a molecular and supramolecular view of the situation for the first time.

In the case of the hydroxyferrocifens, we can differentiate between two behaviour trends depending on the type of receptor concerned: one resembling the antagonist behaviour of hydroxytamoxifen, acting through estrogen receptor alpha, and the other involving the beta receptor, which activates the attached organometallic function. These mechanistic hypotheses are discussed in greater detail below.

Mechanistic hypothesis for the action of the hydroxyferrocifens through estrogen receptor alpha: The hydroxyferrocifens show good to moderate affinity for ERα, depending on the length of the basic chain. The affinity is always lower than that of hydroxytamoxifen, no doubt because of the greater steric hindrance caused by the ferrocenyl group than by a phenyl group (see below). In those cases in which it was possible to separate the *Z* and *E* diastereoisomers in the hydroxyferrocifen series, the *Z* form shows better recognition for the receptor. This can be logically visualised by a molecular modelling study of a hydroxyferrocifen in the active site of the receptor. For the sake of clarity, we show here only the results obtained with (*Z*)-**3b** and (*E*)-**3b** (three-atom side chain), which can be compared to the results reported by Shiau et al. for (*Z*)-hydroxytamoxifen.[13]

MacSpartan Pro Software was used for the molecular modelling studies.[43] Only the amino acids that make up the wall of the binding pocket were retained. The hydroxytamoxifen was removed and replaced successively by the two isomers (*Z*)- and (*E*)-**3b** (Figure 6). The energetically optimal position for the complex was determined with all the heavy atoms of the cavity immobilised, after which the side chain of amino acid His524 was freed. This was justified by the fact that this part of the cavity has been shown to be flexible.[44] An energy minimisation was then carried out with all the heavy (i.e., non-hydrogen) atoms immobilised, except for those of the ligand and the His524 side chain, by use of the Merck Molecular Force Field (MMFF). This determined the ideal position for the ligand. The affinity of the mediator for the cavity was then determined by semiempirical quantum mechanical PM3 calculations. This requires calculation of the energies of the combined ligand and cavity, and of the cavity and the ligand separately, each in the conformation it had in the ligand–cavity combination. This permits measurement of the $\Delta_r H^\circ$ enthalpy variation of the reaction ligand + cavity → ligand–cavity combination. For the (*Z*)-**3b** isomer, the enthalpy variation is $-27.3\ \mathrm{kcal\,mol^{-1}}$, an exothermic value which favours association. For the (*E*)-**3b** isomer, the enthalpy variation is $-21.3\ \mathrm{kcal\,mol^{-1}}$, a value that, while somewhat lower, still favours association. The interior of the active site is thus large enough to accommodate either of these molecules, at their respective volumes of 572 $Å^3$ and 568 $Å^3$, both considerably higher than the 413 $Å^3$ volume of hydroxytamoxifen. This can be seen in Figure 6, which shows the two molecules inside the cavity. Here the positioning of an antagonist organometallic complex in the active site of the receptor can be visualised. It shows the possibility of stabilising interactions between His524 and the ferrocenyl group and between Asp351 and the nitrogen of the basic chain, the latter disappearing when the chain is lengthened. Effects similar to those of hydroxytamoxifen are therefore to be expected when hydroxyferrocifen is acting by the ERα pathway. The presence of the ferrocenyl group, a neutral lipophile, favours entry of the molecule into the cell. The volume of ferrocenyl is greater than that of a phenyl group but binding, with allowance for a few structural adjustments in the details, is very similar to that of hydroxytamoxifen. There is no reason to invoke any other mechanism than that of the conforma-

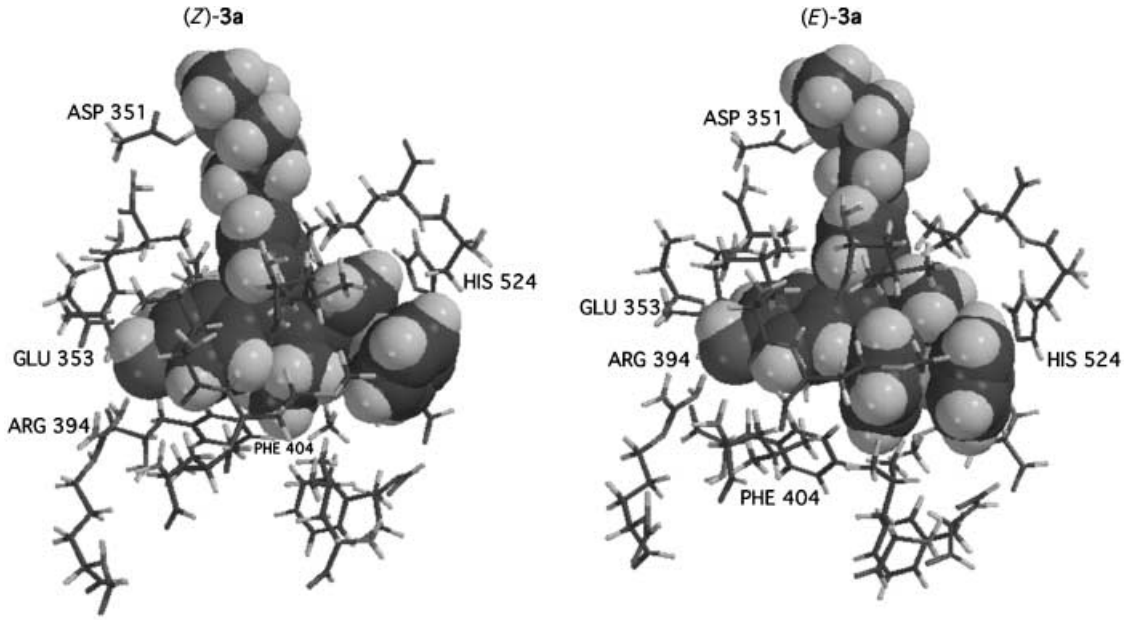


Figure 6. Isomers (*Z*)-**3a** and (*E*)-**3a** as space-filling models in the binding site of the antagonist form of the ERα as a basis for the structure previously described by Shiau et al.[13]

tional change in the ligand binding domain as seen with hydroxytamoxifen, in which the basic chain displaces helix 12 of the receptor, hindering coactivator fixation and thus preventing them from playing their role in activation.

Mechanistic hypothesis for activation of hydroxyferrocifens through estrogen receptor beta: The explanation given above cannot in any way account for the difference in behaviour between hydroxytamoxifen and the hydroxyferrocifens as seen in the MDA-MB 231 cell line. This cell line contains no ERα, but the presence of ERβ has been observed.[41]

The roles of this form of the receptor are still under study and have not yet been fully elucidated. Among the functions that have been reported for ERβ, its possible role in the control of intracellular oxidoreduction particularly attracted our interest[45] since the ferrocene entity is itself easily oxidised to a ferricinium cation radical. This Fe^{2+} to Fe^{3+} conversion in biological media can be compared to the Fenton and Haber–Weiss reactions,[46] which give $O_2^{-\cdot}$ and $OH^{\cdot}$.

It is known that the superoxide radical ion $O_2^{-\cdot}$ is not very reactive with DNA but that the $OH^{\cdot}$ radical is extremely reactive and provokes various types of lesions, making these radicals highly genotoxic. Genotoxicity has recently been shown in the hydroxyferrocifens, while this is not observed with tamoxifen.[27] Other studies have shown that the ferrocifens' ability to cut DNA is due to the generation of ferricinium in situ, and thence of $OH^{\cdot}$ to produce a cytotoxic effect.[23, 47, 48] The fact that transcriptional regulation of the quinone reductase gene can occur preferentially through ERβ bound to an antiestrogen is not the only consideration in evaluation of the oxidoreducing properties of these species.[42] Other authors have in fact observed that the derivatives of tamoxifen are able to induce oxidative stress on human cancer cells classed as ER−, leading to apoptosis.[49] With purely organic molecules this effect only arises at concentrations of 10 μM. It is possible that the increased sensitivity to oxidation (as shown by electrochemistry[27]) of the hydroxyferrocifens activates and enhances the process in cells of this type at levels as low as 1 μM. This would place these molecules in a concentration range compatible with potential therapeutic application, and gives them a clearly novel character combining an antagonist hormonal effect and a cytotoxic function.

The above argument, based at this stage, of course, only on a set of assumptions, militates strongly in favour of the suggestion that in ERβ-containing cell lines a cytotoxic effect is induced by oxidation of the ferrocenyl targeted to the site.

It follows that the hydroxyferrocifens can show two different modes of activity depending on whether ERα or ERβ is involved. This dual behaviour, not possessed to the same extent by hydroxytamoxifen, provides these novel organometallic SERMs, the hydroxyferrocifens, with a wider range of potential applications than tamoxifen, since one or other of their two possible mechanisms of activity will be triggered in response to the specific situation, and at therapeutically appropriate concentrations.

It should be stressed that at this point we have only a plausible hypothesis for the behaviour, and not a proven mechanism. There are in fact other possible routes of activity for these compounds that remain to be explored. Other non-hormone-dependent effects that may contribute to the therapeutic activity of these organometallic molecules include calmodulin antagonism, inhibition of protein kinase C, and growth factor regulation. All these targets have been implicated in the complex activity of tamoxifen and its derivatives.[3, 16, 50] In particular, the chain length of idoxifene and neighbouring series appears to be important in modulation of calmodulin antagonism.[9]

Conclusion

Although other mechanisms may contribute to the overall behaviour of the hydroxyferrocifens, it is certain that the combined antiestrogenic and cytotoxic activity of these compounds as seen in this study appears highly promising in the search for SERMs with better effectiveness than those currently available. This is particularly so in light of acute toxicity studies carried out with hydroxyferrocifen **3b**, showing that this molecule is less toxic in vivo than tamoxifen (lethal dose for rats: 18.9 mg Kg^{-1} (TAM: 7.4 mg Kg^{-1}), for mice: 67.2 mg Kg^{-1} (TAM: 23.7 mg Kg^{-1})). The hydroxyferrocifen molecules with $n = 3$ or 4 appear the best candidates for future commercial development

Experimental Section

General remarks: The synthesis of all the compounds was performed under an argon atmosphere, by use of the Schlenk line technique and Schlenk flasks. Anhydrous THF and anhydrous diethyl ether were obtained by distillation from sodium/benzophenone. TLC chromatography was performed on silica gel 60 GF254. Infrared spectra were obtained on a IR-FT BOMEM Michelson-100 spectrometer equipped with a DTGS detector. ^1H and ^{13}C NMR spectra were recorded on 200 MHz and 250 MHz Bruker spectrometers. Mass spectrometry was performed with a Nermag R 10–10C spectrometer. Melting points were measured with a Kofler device. Elemental analyses were performed by the regional microanalysis service of the Université Pierre et Marie Curie. Ferrocenyl ethyl ketones, **2** and **4a** were prepared by the procedure described in reference [29]. The synthesis of **1**, **3b** and **5b** have been described in reference [26].

1-[4-(2-Dimethylaminoethoxy)phenyl]-1-(4-hydroxyphenyl)-2-ferrocenyl-1-butene (*Z*)-3a and (*E*)-3a: Compound **1** (0.450 g, 10.6 mmol), dissolved in ethanol (10 mL), was added to a solution of sodium ethanolate, prepared by treatment of sodium (0.120 g, 5.2 mmol) with ethanol (20 mL). After the mixture had been stirred for 1 h at 80 °C, 2-chloroethyl-dimethylamine hydrochloride (0.302 g, 2.1 mmol) was added and the mixture was heated under reflux for 3 h. After hydrolysis with water, extraction in diethyl ether and solvent removal, the crude product was chromatographed on silica gel plates with Et_3N/chloroform 1:9 as eluent to give first a mixture of (*Z*)-**3a** and (*E*)-**3a** (0.280 g, 53 % yield). Characteristics of the mixture: mass spectrometry (EI, 70 eV): m/z 495 $[M]^+$, 121 $[CpFe]^+$, 72 $[C_4H_{10}N]^+$, 58 $[C_3H_8N]^+$; elemental analysis calcd (%) for $C_{30}H_{33}NO_2Fe$: C 72.73, H 6.71, N 2.83; found: C 71.02, H 6.72, N 2.77.

The second, more polar fraction corresponds to the product 1,1-bis-[4-(2-dimethylaminoethoxy)phenyl]-2-ferrocenyl-1-butene (**5a**, 0.050 g, 9.5 %). The mixture of the two isomers **1** and **2** was redissolved in a diethyl ether/hexane mixture in the proportions 5:1 and the resulting solution was refrigerated overnight. The *E* isomer (m.p. 93–94 °C) crystallised first after gentle evaporation of the solvent. The crystals of the *E* isomer were isolated and the mother liquor was stored in the freezer for one day. The *Z* isomer (m.p. 181 °C) then crystallised in the form of fine needles. Spectroscopic data: ^1H NMR of compound (*Z*)-**3a** (200 MHz, [D$_6$]DMSO): $\delta = 9.34$ (s, 1H; OH), 6.97 and 6.71 (2 × d, $J = 8,7$ Hz, 2 × 2H; C_6H_4–OH), 6.89 and 6.80 (2 × d, $J = 8.5$ Hz, 2 × 2H; C_6H_4–OCH_2), 4.11 (s, 5H; C_5H_5),

4.07 (m, 2H; C_5H_4), 3.99 (t, $J = 6{,}0$ Hz, 2H; O−CH_2), 3.80 (m, 2H; C_5H_4), 2.60 (t, $J = 6.0$ Hz, 2H; N−CH_2), 2.49 (partially obscured by signals from DMSO, 2H; −CH_2−CH_3), 2.20 (s, 6H; NMe_2), 0.98 ppm (t, $J = 7.3$ Hz, 3H; −CH_2−CH_3). ^{1}H NMR of compound (*E*)-**3a** (200 MHz, [D$_6$]DMSO): $\delta =$ 9.29 (s, 1H; OH), 7.08 and 6.89 (2 × d, $J = 8.5$ Hz, 2 × 2H; C_6H_4−OCH_2), 6.80 and 6.63 (2 × d, $J = 8.4$ Hz, 2 × 2H; C_6H_4OH), 4.11 (s, 5H; C_5H_5), 4.08 (m, 2H; C_5H_4), 4.03 (t, $J = 6{,}0$ Hz, 2H; O−CH_2), 3.82 (m, 2H; C_5H_4), 2.64 (t, $J = 6.0$ Hz, 2H; N−CH_2), 2.49 (partially obscured by signals from DMSO, 2H; −CH_2−CH_3), 2.23 (s, 6H; NMe_2), 0.98 ppm (t, $J = 7.3$ Hz, 3H; −CH_2−CH_3). ^{1}H NMR of compound **5a** (200 MHz, $CDCl_3$): $\delta = 7.10$ and 6.75 (2 × d, $J = 8.8$ Hz, 4H; C_6H_4), 6.95 and 6.87 (2 × d, $J = 8.7$ Hz, 4H; C_6H_4), 4.10 (s, 5H; C_5H_5), 4.06 (m, 6H; C_5H_4 and 2 × O−CH_2), 3.89 (m, 2H; C_5H_4), 2.76 (m, 4H; 2 × N−CH_2), 2.57 (q, $J = 7.45$ Hz, 2H; −CH_2−CH_3), 2.38 and 2.36 (2 × s, 12H; 2 × NMe_2), 1.01 ppm (t, $J = 7.5$ Hz, 3H; −CH_2−CH_3).

[4-(4-Bromopentoxy)phenyl] (4-hydroxyphenyl) ketone 6c: Dihydroxybenzophenone (2.14 g, 10 mmol) was added to a solution of sodium ethanolate prepared by treatment of sodium (0.230 g, 10 mmol) with ethanol (15 mL). After the mixture had been stirred at reflux for 1 h, dibromobutane (11.45 g, 50 mmol) was added. After 1 h at reflux, the solution was left to cool to room temperature and hydrolysed with water (100 mL). The product was extracted with dichloromethane. The organic phase was washed with water, dried over magnesium sulfate and filtered, and the solvent was evaporated. The crude product was chromatographed on silica gel plates with diethyl ether/pentane 2:1 as eluent. Compound **6c** (1.405 g, 39%) was isolated. m.p. 86 °C; ^{1}H NMR (200 MHz, [D$_6$]acetone): $\delta =$ 7,74 and 7.05 (2 × d, 2 × 2H; C_6H_4−O), 7,69 and 6.96 (2 × d, 2 × 2H; C_6H_4−OH), 4,13 (t, 2H; OCH_2), 3,54 (t, 2H; CH_2Br), 1.90 and 1.66 ppm (2 × m, 6H; $(CH_2)_3$).

[4-(4-Chlorooctoxy)phenyl] (4-hydroxyphenyl) ketone 6d: The procedure was similar to that used for **6c**, with sodium (0.115 g, 5 mmol), dihydroxybenzophenone (1.07 g, 5 mmol), 1,8-dichlorooctane (4.580 g, 25 mmol). After workup, the crude product was chromatographed on silica gel plates with ethyl ether/pentane 2:1 as eluent. Compound **6e** (0.620 g, 34.4%) was isolated as a white solid. m.p. 99 °C; ^{1}H NMR (200 MHz, [D$_6$]acetone): $\delta =$ 7,74 and 7.04 (2 × d, 2 × 2H; C_6H_4−O), 7.68 and 6.96 (2 × d, 2 × 2H; C_6H_4−OH), 4.11 (t, 2H; OCH_2), 3.60 (t, 2H; CH_2Cl), 1.79 and 1.47 ppm (2 × m, 4H, 8H, $(CH_2)_6$).

[4-(4-Chlorooctoxy)phenyl] (phenyl) ketone 7d: The procedure was similar to that used for **6c**, with sodium (0.230 g, 10 mmol), hydroxybenzophenone (0.99 g, 5 mmol), 1,8-dichlorooctane (4.580 g, 25 mmol). After workup, the crude product was chromatographed on silica gel plates with ethyl ether/pentane 1:4 as eluent. Compound **7d** (0.820 g, 47.6%) was isolated as a white solid. m.p. 56 °C; ^{1}H NMR (200 MHz, [D$_6$]acetone): $\delta =$ 7,81 – 7.53 (m, 9H; C_6H_5 + C_6H_4), 4.12 (t, 2H; OCH_2), 3.60 (t, 2H; CH_2Cl), 1.79 and 1.41 ppm (2 × m, 4H, 8H; $(CH_2)_6$).

1-[4-(4-Bromopentoxy)phenyl]-1-(4-hydroxyphenyl)-2-ferrocenyl-but-1-ene (*Z* + *E*)-9c: The procedure was similar to that used for **1**, with $TiCl_4$ (0.346 g, 1,82 mmol), zinc (0,235 g, 3,60 mmol), **6c** (0.225 g, 0,6 mmol), ferrocenyl ethyl ketone (0.145 g, 0,6 mmol). After workup, the crude product was chromatographed on silica gel plates with ethyl ether/pentane 1:2 as eluent. Compound **9c** (0.175 g, 50.7%) was isolated as an oil. ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 6.27 – 6.65$ (m, 8H; 2 × C_6H_4), 4.11 and 4.10 (2 × s, 5H; C_5H_5), 4.07 (t, 2H; C_5H_4), 3,97 and 3.92 (2 × m, 2 × 2H; $C_{55}H_{44}$ and OCH_{22}), 3,45 and 3.44 (2 × t, 2H; CH_2Br), 2,58 (q, 2H; CH_2CH_3), 1.92, 1.83, 1.69 (3 × m, 3 × 2H; $(CH_2)_3$), 1,02 ppm (t, 3H; CH_2CH_3).

1-[4-(4-Bromopentoxy)phenyl]-1-(phenyl)-2-ferrocenyl-but-1-ene (*Z* + *E*)-10c: Compound **2** (0.41 g, 1 mmol) was dissolved in acetone (20 mL), powdered NaOH (0.080 g, 2 mmol) was added, and the mixture was heated at reflux for 1.5 h. Dibromopentane (0.28 g, 2 mmol) was then added. After being heated for 2 h, the solution was left to cool to room temperature and was then hydrolysed with water (100 mL). The product was extracted with ether (2 × 60 mL). The organic phase was washed with water, dried over magnesium sulfate and filtered, and the solvent was evaporated. The crude product was chromatographed on silica gel plates with diethyl ether/pentane 1:10 as eluent to afford **10c** (*Z* + *E*) in 50% yield (0.27 g). ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 7.11$ (m, 9H; C_6H_5 and C_6H_4), 4.11 (s, 5H; C_5H_5), 4.06 and 3.87 (2 × m, 4H; C_5H_4), 3.93 (m, 2H; OCH_2), 3.44 (m, 2H; CH_2Br), 2.61 and 2.57 (2 × q, 2H; CH_2CH_3), 1.87 and 1.59 (2 × m, 4H, 2H, $(CH_2)_3$), 1.02 and 1.04 ppm (2 × t, 3H; CH_2CH_3).

1-[4-(4-Chlorooctoxy)phenyl]-1-(4-hydroxyphenyl)-2-ferrocenyl-but-1-ene (*Z* + *E*)-9d: The procedure was similar to that used for **9c**, with $TiCl_4$ (0.7 mL, 6 mmol), zinc (0.780 g, 12 mmol), **6e** (0.720 g. 0.6 mmol), ferrocenyl ethyl ketone (0.485 g, 2 mmol). After workup, the crude product was chromatographed on silica gel plates with ethyl ether/pentane 1:3 as eluent to afford **9e** (0.531 g, 46.5% yield). ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 7.13 –$ 6.65 (m, 8H; 2 × C_6H_4), 4.73 (s, 1H; OH), 4.11 (s, 5H; C_5H_5), 4.06 and 3.92 (2 × m, 2 × 2H; C_5H_4), 3,95 (m, 2H; OCH_2), 3,55 and 3.54 (2 × t, 2H; CH_2Cl), 2,59 (q, 2H; CH_2CH_3), 1.80 and 1.39 (2 × m, 4H, 8H, $(CH_2)_6$), 1,02 ppm (t, 3H; CH_2CH_3).

1-[4-(4-Chlorooctoxy)phenyl]-1-(phenyl)-2-ferrocenyl-but-1-ene (*Z* + *E*)-10d: The procedure was similar to that used for **9c**, with $TiCl_4$ (0.7 mL, 6 mmol), zinc (0.780 g, 12 mmol), **6d** (0.690 g, 2 mmol) and ferrocenyl ethyl ketone (0.484 g, 2 mmol). After workup, the crude product was chromatographed on silica gel plates with pentane as eluent to afford **10d** (0.435 g, 39%) as an oil. ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 7.32 – 6.73$ (m, 9H; C_6H_5 and C_6H_4), 4.11 (s, 5H; C_5H_5), 4.07 (m, 2H; C_5H_4), 3,90 (m, 4H; C_5H_4 and OCH_2), 3.55 and 3.54 (2 × t, 2H; CH_2Cl), 2,60 and 2.57 (2 × q, 2H; CH_2CH_3), 1.79 and 1.40 (2 × m, 4H; 8H; $(CH_2)_6$), 1.04 and 1.03 ppm (2 × t, 3H; CH_2CH_3); MS (EI): *m*/*z*: 554 $[M]^+$, 489 $[M - Cp]^+$, 407, 378, 343, 327.

1-[4-(4-Dimethylaminopentoxy)phenyl]-1-(4-hydroxyphenyl)-2-ferrocenyl-but-1-ene (*Z* + *E*)-3c: (*Z* + *E*)-1-[4-(4-Bromopentoxy)phenyl]-1-(4-hydroxyphenyl)-2-ferrocenyl-but-1-ene (**9c**, 0.145 g, 0.25 mmol), $HNMe_2$·HCl (0.210 g, 2.5 mmol), NEt_3 (0.2 mL) and ethanol (25 mL) were introduced into an autoclave. The mixture was heated at 110 °C for 6 h. The solvent was evaporated. The crude product obtained was chromatographed on silica gel plates with acetone/NEt_3 20:1 as eluent to afford **3c** (0.115 g, 85.7%). ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 7.07 – 6.63$ (m, 8H; 2 × C_6H_4), 4.10 (s, 5H; C_5H_5), 4.06 (t, 2H; C_5H_4), 3.91 (m, 4H; OCH_2 and C_5H_4), 2.56 (q, 2H; CH_2CH_3), 2.30 (m, 2H; −CH_2NMe_2), 2.27 and 2.26 (2 × s, 6H; NMe_2), 1.80 (m, 2H; −CH_2), 1.50 (m, 4H; $(CH_2)_2$), 1.02 ppm (t, 3H; CH_2CH_3); MS (EI): *m*/*z*: 537 $[M]^+$, 220, 205, 149, 114; elemental analysis calcd (%) for $C_{33}H_{39}NO_2Fe \cdot 2H_2O$: C 69.11, H 7.55, N 2.44; found: C 69.44, H 7.28, N 2.43.

1-[4-(4-Dimethylaminopentoxy)phenyl]-1-(phenyl)-2-ferrocenyl-but-1-ene (*Z* + *E*)-4c: The procedure was similar to that used for **3c**, with **10c** (0.480 g, 0.86 mmol), $HNMe_2$·HCl (0.760 g, 8.6 mmol), NEt_3 (0.5 mL) and ethanol (25 mL). After solvent removal, the crude product was chromatographed on silica gel plates with NEt_3/acetone 1:20 as eluent to afford **4c** (0.280 g, 62%). ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 6.99$ (m, 9H; C_6H_4 and C_6H_5), 4.11 (s, 5H; C_5H_5), 3.93 (m, 3H; OCH_2 and 1H from C_5H_4), 2.60 and 2.57 (2 × q, 2H; CH_2CH_3), 2.30 (m, 2H; −CH_2NMe_2), 2.27 and 2.26 (2 × s, 6H; NMe_2), 1.82 (m, 2H; −CH_2), 1.53 (m, 4H; $(CH_2)_2$), 1.06 and 1.04 ppm (2 × t, 3H; CH_2CH_3).

1-[4-(4-Dimethylaminooctyloxy)phenyl]-1-(4-hydroxyphenyl)-2-ferrocenyl-but-1-ene (*Z* + *E*)-3d: The procedure was similar to that used for **3c**, with **9d** (0.420 g, 0.74 mmol), $HNMe_2$·HCl (0.600 g, 7.4 mmol) and NEt_3 (0.5 mL). After solvent removal, the crude product was chromatographed on silica gel plates with NEt_3/acetone 1:20 as eluent to afford **3d** (0.180 g, 42.3%). M.p. 70 °C; ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 7.12 – 6.62$ (m, 8H; 2 × C_6H_4), 4.10 (s, 5H; C_5H_5), 4.06 (m, 2H; C_5H_4), 3.90 (m, 4H; OCH_2 and C_5H_4), 2.59 (q, 2H; CH_2CH_3), 2.28 (m, 2H; −CH_2NMe_2), 2.26 (s, 6H; NMe_2), 1.72 and 1.26 (2 × m, 12H; $(CH_2)_6$), 1.06 and 1.02 ppm (2 × t, 3H; CH_2CH_3); MS (EI): *m*/*z*: 579 $[M]^+$, 359, 343, 220, 205; elemental analysis calcd (%) for $C_{36}H_{45}NO_2Fe$: C 74.60, H 7.82, N 2.41; found: C 74.56, H 7.87, N 2.55.

1-[4-(4-Dimethylaminooctyloxy)phenyl]-1-(phenyl)-2-ferrocenyl-but-1-ene (*Z* + *E*)-4d: The procedure was similar to that used for **3c**, with **10d** (0.265 g. 0.48 mmol), $HNMe_2$·HCl (0.390 g, 7.4 mmol) and NEt_3 (0.3 mL). After solvent removal, the crude product was chromatographed on silica gel plates with NEt_3/acetone 1:20 as eluent to afford **4d** (0.170 g, 63%) as an orange oil. ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 7.32 – 6.72$ (m, 9H; C_6H_5 and C_6H_4), 4.11 (s, 5H; C_5H_5), 4.07 and 3.87 (2 × m, 2 × 2H; C_5H_4), 3.87 (t, 2H; OCH_2), 2.58 (m, 2H; CH_2CH_3), 2.24 (m, 2H; −CH_2NMe_2), 2.22 (s, 6H; NMe_2), 1.77 and 1.26 (2 × m, 12H; $(CH_2)_6$), 1.04 and 1.02 ppm (2 × t, 3H; CH_2CH_3); MS (EI): *m*/*z*: 563 $[M]^+$, 343 , 327, 207.

Ethyl ferrocenylacetate 12: Concentrated H_2SO_4 (0.5 mL) was added to a solution of ferrocenylacetic acid (2.00 g, 8.2 mmol) in ethanol (50 mL). The

mixture was heated at reflux for 1 h. The solution was left to cool to room temperature and hydrolysed with water (100 mL). The product was extracted with diethyl ether (3 × 40 mL). The organic phase was washed with water, dried over magnesium sulfate and filtered, and the solvent was evaporated to afford **12** (2.22 g). The crude product was used without further purification. ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 4.23$ and 4.13 (2 × m, 2 × 2H; C_5H_4), 4.18 (q, 2H; OCH_2), 4.14 (s, 5H; C_5H_5), 3.34 (s, 2H; CH_2), 1.31 ppm (t, 3H; Me).

1,1-Bis(4-methoxyphenyl)-2-ferrocenylethene 13: Iodoanisole (1.123 g, 4.80 mmol) was dissolved in dry diethyl ether (10 mL). This solution was cooled to 0 °C and a solution of *n*BuLi in hexane (1.6 M, 2.5 mL, 4.00 mmol) was then added dropwise by dropping funnel. After completion of addition, stirring was maintained for 15 min more while the temperature was kept at 0 °C. A solution of ethyl ferrocenylacetate **12** (0.408 g, 1.5 mmol) in dry ether (15 mL) was added slowly to the first solution. After 2 h of stirring, during which the temperature was allowed to rise to room temperature, the solution was hydrolysed with HCl (1:10, 30 mL). The mixture was heated at reflux for 4 h. After cooling to room temperature, the product was extracted with diethyl ether and the organic phase was washed with water. After drying over magnesium sulfate the solution was filtrated and concentrated with a rotary evaporator. The crude product was chromatographed on silica gel plates with diethyl ether/pentane 1:9 as eluent to afford **13** (0.325 g, 51 %) as an orange oil. ^{1}H NMR (200 MHz, $CDCl_3$): $\delta =$ 7.22 and 6.84 (2 × d, 2 × 2H; C_6H_4), 7.15 and 6.94 (2 × d, 2 × 2H; C_6H_4), 6.62 (s, 1H; =CH), 4.15 (m, 7H; C_5H_5 and 2H of C_5H_4), 3.91 (s, 2H; Cp and C_5H_4), 3.88 and 3.82 ppm (2 × s, 2 × 3H; 2 × OMe).

1,1-Bis(4-hydroxyphenyl)-2-ferrocenylethene 14: Compound **13** (0.120 g, 0.26 mmol) was dissolved in dry CH_2Cl_2 (8 mL). The solution was cooled to −78 °C, and a BBr_3 solution (1 M in CH_2Cl_2, 1.06 mL) was then added dropwise. The cooling bath was removed and the solution was stirred for 30 min. The solution was poured into iced water (100 mL). After the mixture had been stirred for 10 min, NaCl was added to saturate the solution. The product was extracted with diethyl ether (4 × 30 mL). The organic phase was washed with water, dried over $MgSO_4$ and filtrated. After solvent removal, the crude product was chromatographed on silica gel plates with acetone/pentane 2:3 as eluent to afford **14** (0.065 g, 62 %) as an orange solid. m.p. 72 °C; ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 7.20$ and 6.77 (2 × d, 2 × 2H; C_6H_4), 7.11 and 6.89 (2 × d, 2 × 2H; C_6H_4), 6.63 (s, 1H; =CH), 5.29 and 5.26 (2 × s, 2 × 1H; 2 × OH), 4.12 (s, 5H; C_5H_5), 4.09 and 3.86 ppm (2 × m, 2 × 2H; C_5H_4); elemental analysis calcd (%) for $C_{24}H_{20}O_2Fe$: C, 72.74, H 5.09; found: C 72.73, H 5.44.

1-[4-(2-Dimethylaminoethoxy)phenyl]-1-(4-hydroxyphenyl)-2-ferrocenylethene 15: A solution of sodium ethanolate was prepared by treating sodium (0.138 g, 6 mmol) with ethanol (10 mL). Compound **14** (0.100 g, 0.25 mmol) was added to this solution. After the mixture had been stirred for 1 h, 2-dimethylaminoethyl chloride hydrochloride (0.576 g, 4 mmol) was added. After 4 h of heating, the solution was left to cool to room temperature and then hydrolysed with water (20 mL). The product was extracted with ether. The organic phase was washed with water, dried over magnesium sulfate and filtered, and the solvent was evaporated. The crude product was chromatographed on silica gel plates with methanol/chloroform 30:70 as eluent to give first a mixture of two isomers of **15** (0.048 g, 41 % yield). ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 7.25-6.78$ (m, 8H; 2 × C_6H_4), 6.62 (s, 1H; =CH), 4.12 (s, 5H; C_5H_5), 4.26 (m, 2H; OCH_2), 4.09 and 3.86 (2 × m, 2 × 2H; C_5H_4), 3.17 (m, 2H; N−CH_2), 2.74 and 2.71 ppm (2 × s, 6H; NMe_2).

The second fraction was identified as **16** (0.035 g, 30 %). ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 7.18$ and 6.81 (2 × d, 2 × 2H; C_6H_4), 7.10 and 6.97 (2 × d, 2 × 2H; C_6H_4), 6.63 (s, 1H; =CH), 4.12 (s, 5H; C_5H_5), 4.09 (m, 4H; 2 × OCH_2), 4.09 and 3.84 (2 × m, 2 × 2H; C_5H_4), 3.21 (m, 4H; 2 × N−CH_2), 2.74 and 2.70 ppm (2 × s, 2 × 6H; 2 × NMe_2).

X-ray crystal structure determination for (*E*)-3a: A suitable crystal of (*E*)-**3a** crystallised from acetone in the monoclinic space group $P2_1/c$. Accurate cell dimensions and orientation matrices were obtained by least-squares refinements of 25 accurately centred reflections. No significant variations were observed in the two check reflections during data collections. The data were corrected for Lorentz and polarisation effects; an empirical absorption (DIFABS) was applied.[51] Computations were performed by use of the PC version of CRYSTAL.[52] Scattering factors and corrections for anomalous absorption were taken from [51].

Biochemical materials and methods: 17β-Estradiol and protamine sulfate (from salmon grade X) and hydroxytamoxifen ($Z + E$) were obtained from Sigma. (*Z*)-Hydroxytamoxifen was a gift from Besins Isovesco (France). [6,7-^{3}H]-17β-estradiol (SA: 1.628 TBq mmol^{-1}) was from Perkin Elmer Life Sciences.

Animal tissues: Lamb uteri weighing approximately 7 g were obtained from the slaughterhouse at Mantes-la-Jolie, France. They were immediately frozen and kept in liquid nitrogen prior to use.

Determination of the relative binding affinities (RBAs) of the compounds for the alpha and beta forms of the estrogen receptor

Preparation of the stock solutions of ferrocifens: Stock solutions (1×10^{-3} M) of the ferrocene derivatives were prepared in absolute ethanol or DMSO. They are stable for at least one month provided they are kept in the dark at −20 °C (ethanol solutions) or 4 °C (DMSO solutions)

Sheep uterine cytosol prepared in buffer A (0.05 M Tris-HCl, 0.25 M sucrose, 0.1 % β-mercaptoethanol, pH 7.4 at 25 °C) as described in reference [53] was used as a source of ERα. The beta form of the receptor, produced in a baculovirus-mediated expression system, was purchased from PanVera (USA). Typically, 10 μL of estrogen receptor beta (3500 pmol mL^{-1}) was added to 16 mL of buffer B (10 % glycerol, 50 mM bis-tris-propane pH 9, 400 mM KCl, 2 mM DTT, 1 mM EDTA, 0,1 % BSA) in a silanised flask. Aliquots (200 μl) of ERα (in glass tubes) or ERβ (in propylene tubes) were incubated for 3 h at 0 °C or 25 °C with 2×10^{-9} M of [6,7-^{3}H]-17β-estradiol in the presence of nine concentrations of the compounds to be tested (nine concentrations prepared just prior to use from the 1×10^{-3} M stock solution in absolute ethanol or DMSO; final volume of ethanol or DMSO equal to 5 %). A the end of the incubation period the free and bound fractions of the tracer were separated by protamine sulfate precipitation as follows. An equal volume of phosphate buffer containing 1 mg mL^{-1} of protamine sulfate was added to each tube. The mixtures were vortexed and allowed to stand at 0 °C for 10 min. The resulting precipitates were filtered on glass fibre paper (Watman GF/C) under a moderate vacuum and washed with ice-cold phosphate buffer (40 mL). The filters were then transferred to scintillation vials, and radioactivity was measured on a LKB-1211 RackBeta counter. As hydroxyapatite was used in the literature instead of protamine sulfate to separate the free and bound fraction of the tracer we have checked that the RBA values obtained by both methods are equivalent. The percentage reduction in binding of [^{3}H]-estradiol (Y) was calculated by the use of the logit transformation of Y [logit Y = $\ln[\langle BY/(1-Y)\rangle]$] versus the log of the mass of the competing steroid. The concentration of unlabelled steroid required to displace 50 % of the bound [^{3}H]-estradiol (logit Y = 0) was calculated for each steroid tested, and the results were expressed as RBA. The RBA value of estradiol is by definition equal to 100 %

Culture cells: MCF7 and MDA-MB231 cells were from the Michigan Cancer Foundation (Detroit), RTx6[54] and TD5,[55] two MCF7 mutants selected for their resistant to tamoxifen, were from J.C. Faye (INSERM U168, Toulouse, France) and F. Van Roy (RUG, Ghent, Belgium). Earle's minimal essential medium (MEM), foetal bovine serum (FBS), L-glutamine, penicillin, gentamicin and streptomycin were obtained from Gibco (Ghent, Belgium), plastic culture materials from Falcon (Ghent, Belgium)

Culture conditions: Cells were maintained in monolayer culture in Dulbecco-MEM with 10 % added thermally inactivated FBS, L-glutamine (0.6 mg mL^{-1}) and a cocktail of antibiotics (gentamicin 40 μg mL^{-1}, penicillin 100 U mL^{-1}, streptomycin 100 μg mL^{-1}). The growth of the cells was assessed by measuring the DNA content of treated and untreated (control) cells after 120 h of culture.[56]

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