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The Presence of a Ferrocenyl Unit on an Estrogenic Molecule is Not Always Sufficient to Generate in vitro Cytotoxicity

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We recently reported the dual (antihormonal and cytotoxic) functionality of ferrocifens, which are organometallic complexes derived from hydroxytamoxifen, the standard molecule in the treatment of hormone-dependent breast cancers. To test the hypothesis that the presence of a ferrocenyl substituent on molecules with an affinity for the estrogen receptor is sufficient to give them cytotoxic properties in vitro, we prepared complexes derived from estradiol with a ferrocenyl substituent at positions 7α and 17α. The complexes thus obtained retain a satisfactory level of affinity for the estrogen receptor (RBA values higher than 12%). At low concentrations (0.1–1 μM) the complexes show an estrogenic effect in vitro equivalent to that of estradiol on hormone-dependent (MCF-7) breast cancer cells, and no cytotoxic effect on hormone-independent (MDA-MB-231) breast cancer cells. At high concentrations (up to 50 μM) the 17α-ethynylferrocenyl estradiol and 7α-ferrocenylmethylthio estradiol become cytotoxic (IC_{50} = 13.2 μM and 18.8 μM, respectively) while the 17α-ferrocenylestradiol remains non toxic. The low toxicity of these compounds support our hypothesis that electronic communication between the ferrocenyl and phenol moieties in the hydroxyferrocifens series is a key parameter in the generation of cytotoxic effects at submicromolar concentrations.

Introduction

For the last few years we have been working with the ferrocifens **1**, a series of organometallic complexes in which the β-phenyl group of hydroxytamoxifen (OH-Tam) **2**, the active metabolite of tamoxifen **3** (the standard drug for adjuvant treatment of hormone-dependent breast cancers) is substituted by a ferrocenyl group[1,2] (Table 1). These complexes are characterized in vitro by a strong antiproliferative effect on both hormone-dependent (MCF-7) and hormone-independent (MDA-MB-231) breast cancer cells (IC_{50} values around 0.5 μM), whereas OH-Tam has an effect only on hormone-dependent cells. The antiproliferative effect of OH-Tam on hormone-dependent cells is traditionally ascribed to an antiestrogenic effect mediated by a specific interaction with the estrogen receptor (ERα). The X-ray crystal structure of the active site of the ligand binding domain of the ERα containing OH-Tam shows that the bulky dimethylamino side chain does not allow a helix of the active site (helix 12 on helix 4) to fold correctly, which prevents fixation of the co-activators and the triggering of the hormonal effect.[3] The antiproliferative effect observed with the ferrocifens, **1**, on hormone-dependent cells appears at first sight to be of the same type. In fact these molecules also possess the dimethylamino chain, which we took care to lengthen slightly (n = 3, 4, and 5 for the most active complexes) to compensate for the increased steric hindrance generated by substitution of the phenyl group by a ferrocenyl group. However, the antiproliferative effect observed on MDA-MB-231 cells, a cell line without ERα, can only be explained as a cytotoxic effect due to the ferrocenyl moiety attached to a hormonal vector. We have also shown that the ferrocenyl-monophenol **4**[1] and particularly the ferrocenyl-diphenol **5**[4] at incubation concentrations of 1 μM, have a clear antiproliferative effect on MCF-7 and MDA-MB-231 cells, whereas **6**, the organic equivalent of **5**, as expected for a molecule with no dimethylamino side chain, has a proliferative effect on MCF-7 cells and no effect on MDA-MB-231 cells. To explain this dual effect of antihormonal and cytotoxic behavior, one could hypothesize that the specific interaction between the organometallic hormone and the estrogen nuclear receptor has permitted iron(II) to be transported into the cell and to be oxidized to ferrocenium ions at, for example, the mitochondrial level. The cytotoxic effect of ferrocenium ions has been previously observed by Köpf-Maier[5,6] and Osella.[7,8] However,

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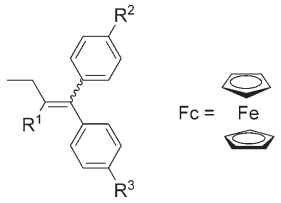
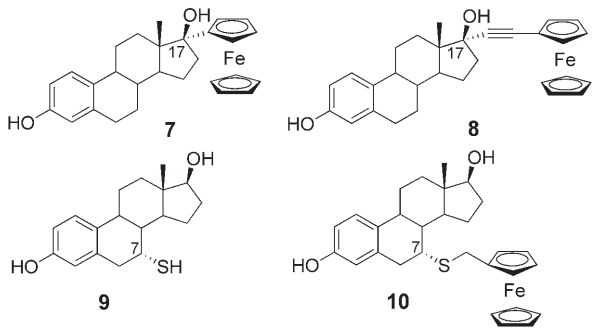
Table 1. Structures of ferrocenyldiphenylethylene and of triphenylethylene derivatives.



Compound	R^1	R^2	R^3
1 (*Z*+*E*)-ferrocifens	Fc	OH	O-$(CH_2)_n$-N$(CH_3)_2$ $n=3$–5
2 (*Z*+*E*)-OH-Tam	Ph	OH	O-$(CH_2)_2$-N$(CH_3)_2$
3 (*Z*+*E*)-Tam	Ph	H	O-$(CH_2)_2$-N$(CH_3)_2$
4 (*Z*+*E*)-Fc-monoOH	Fc	H	OH
5 Fc-diOH	Fc	OH	OH
6 di-OH	Ph	OH	OH

this effect is only obtained at high concentrations (0.01–0.1 mM) as it is difficult for charged species to penetrate the lipid barrier of the cell membrane. Moreover, it has recently been shown that ferrocenium ions are capable of interacting with DNA,[7,9] while ferrocene itself has no effect, despite the fact that its lipophilicity, very similar to that of estradiol (log $P_{o/w}=3.3$ and 3.5 respectively), allows it to enter cells without difficulty.[2] This suggests the hypothesis that the presence of a ferrocenyl substituent on a complex that has affinity for the estrogen receptor allows these lipophilic complexes to be concentrated in estrogen receptor positive cells. They can then be oxidized, generating ferrocenium ions and hence a cytotoxic effect. To test this hypothesis we prepared complexes derived from estradiol and possessing a ferrocenyl group in position 17α (compounds **7** and **8**) or 7α (compound **10**), positions that are known to tolerate the addition of bulky substituents.[10–13] The lipophilicity of these complexes and their affinity for the α form of the estradiol receptor (ERα) was measured, and their effect on proliferation in hormone-dependent (MCF-7) and hormone-independent (MDA-MB-231) cell lines was tested. Molecular modeling of the complexes in the active site of the estradiol receptor, and a study of the electrochemical oxidation of their ferrocenyl group, were also performed. This work is described below.



Results and Discussion

Synthesis of the complexes

7α-ferrocenylmethylthio-17β-estradiol 10

It has been previously shown that ferrocenylmethanol ($FcCH_2OH$) **11** can react with the SH group of cysteine derivatives in THF in the presence of a catalytic amount of TFA (trifluoroacetic acid) to yield the thioether derivative.[14–16] This reaction takes advantage of the facile in situ generation of the highly stabilized α-ferrocenylcarbenium ion **12** in the presence of a strong acid and its subsequent addition to the thiol. The application of this reaction to 7α-thioestradiol **9**, prepared in seven steps as previously described by Napolitano[13] gave **10** in good yield (58%, Scheme 1).

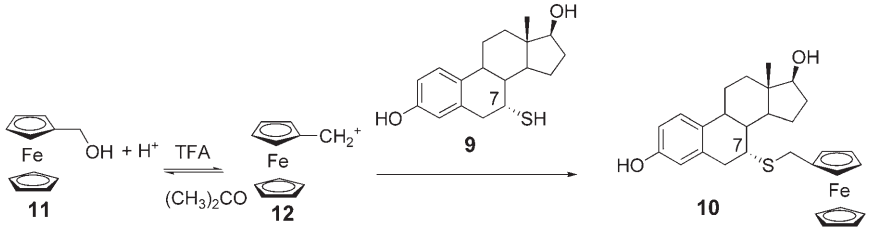
Because of the large number of steps necessary to synthesize products substituted in position 7, only a few 7α-estradiol derivatives have been made[10,12,13,17,18] and there is only one series of metallic (rhenium) complexes with a chelate or an organometallic complex at this position.[12] Complex **10** is, to the best of our knowledge, the first example of an estradiol with a ferrocenyl substituent attached at position 7α.

17α-Ferrocenylethynyl-17β-estradiol derivative **8** was prepared according to the synthesis described by Wenzel.[19] This synthesis was preferred to the one published more recently by Osella.[20] The 17α-ferrocenyl-17β-estradiol **7** was prepared as previously described.[21]

Biochemical studies

Determination of the relative binding affinity (RBA) values for the estrogen receptor and lipophilicity of the complexes

The RBA values of compounds **7**–**10** for the α form of the estrogen receptor (ERα) were measured (Table 2) and compared with the RBA value of estradiol (E_2), the estrogen of reference. All the complexes preserve satisfactory affinity for ERα. This confirms that positions 17α and 7α tolerate the addition of a bulky substituent, here ferrocenyl. In position 17α, the addition of this bulky substituent is better tolerated when it is not too close to the steroid (RBA = 12.5% for **7** and 28% for **8**). In position 7α, addition of the ferrocenyl causes a reduction in the RBA value (RBA = 43% for **9** and 12% for **10**) confirming that



Scheme 1. Synthesis of 7α-ferrocenylmethylthio-17β-estradiol **10**.

Table 2. Relative binding affinity (RBA) values for ERα, lipophilicity ($\log P_{o/w}$), and in vitro proliferative effect on hormone-dependent breast cancer cells (MCF-7) of the compounds.

Compound	RBA [%][a]	$\log P_{o/w}$	Proliferative Effect of X/E_2 [%][e]
17β-estradiol (E_2)	100[b]	3.2	100
7	12.5 ± 2.4[c]	4.7	103
8	28.1 ± 2.7	5.5	83
9	43 ± 0[d]	2.8	95
10	12 ± 1.5	5.0	94

[a] Values represent the mean of two or three experiments; [b] value by definition; [c] measured in silanized tubes; [d] value from ref. [13]; [e] measured on MCF-7 cells in the presence of 1 μM of the compound (X) to be tested or 1 nM of E_2; conditions as described in Figure 1

short chains with bulky substituents at the 7α position attenuate binding affinity.[17]

In terms of lipophilicity, measured by the $\log P_{o/w}$ value, addition of a lipophilic ferrocenyl substituent increases this value whereas addition of an SH decreases it (Table 2). The ferrocenyl complexes have $\log P_{o/w}$ values in the range 4.7–5.5, which appears to be a good compromise that allows the complex to penetrate the cell while retaining a reasonable nonspecific binding rate.[12]

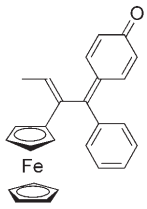
In vitro effect of the complexes on the proliferation of hormone-dependent (MCF-7) and hormone-independent (MDA-MB-231) breast cancer cells

We examined the effect of these complexes on the growth of MCF-7 hormone-dependent cancer cells and compared it to that obtained with estradiol, the standard estrogen (Figure 1). On these cells estradiol had the expected strong proliferative effect (on average 187% relative to control), and at concentrations between 0.1 μM and 1 μM, the ferrocenyl complexes displayed a strong proliferative effect almost equal to that observed with estradiol (Figure 1, Table 2). In all probability this effect is imputable to an estrogenic effect. On MDA-MB-231 hormone-independent breast cancer cells, which do not contain ERα, neither estradiol nor the complexes had any effect at these low concentrations (see Supporting Information). The complexes behaved like estrogens and lacked the cytotoxic effect observed with the ethylene diphenyl complexes **1**, **4**, and **5** at the same concentrations. Here the ferrocene apparently behaves as a bystander substituent analogous to a phenyl group.[22] However, at higher concentrations (up to 50 μM, the limit of solubility of the complexes) **8** and **10** became cytotoxic on MDA-MB-231 cells (IC_{50} = 13.4 μM ± 0.7 for **8** and 18.8 μM ± 0.1) whereas **7** still had no effect.

Electrochemical studies

The cytotoxicity of ferrocene compounds in vitro has been linked to the redox behavior of iron[5,7,9] suggesting the electrochemical analysis of **7**, **8**, and **10.** In MeOH, at scan rates from 0.05 to 20 $V s^{-1}$, each compound gave rise to a conventional, reversible ferrocene/ferrocenium couple. Oxidation potentials of 0.381 V for **7** (see Supporting Information), 0.532 V for **8** and 0.439 V for **10** versus SCE at 0.5 $V s^{-1}$, with broad, irreversible phenol oxidation waves at higher potentials for **7** and **8**, were observed. The addition of pyridine to the complex/MeOH solutions resulted in no qualitative change to the cyclic voltammograms (CVs). Ferrocene oxidation waves were observed at 0.391 V for **7**, 0.556 V for **8**, and 0.439 V for **10** versus SCE, and the phenol oxidation waves (where present) broadened considerably.

We have previously proposed that conjugated quinoid species such as **17** are responsible for the observed cytotoxicity of some ferrocenyl phenol compounds, notably the ferrocenyl diphenol ethylene **5**.[23] Electrochemical results strongly suggest that quinoids can be formed intracellularly according to a pH sensitive mechanism which involves initial ferrocene oxidation followed by pi-system-mediated hole transfer to, and deprotonation of, a phenol functionality. These electrophilic species are highly reactive and could theoretically react with proteins or nucleic acids in the cell.



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This mechanism is consistent with our laboratory observations of an intramolecular chemical reduction of electrochemically produced ferrocenium cations, manifested by the loss of the ferrocenium reduction wave in the CV. This behavior, however, is not observed for the estrogens, **7**, **8**, and **10**; indeed the oxidoreduction waves are essentially unperturbed by the presence of pyridine. The ferrocenium moiety is electronically isolated from the phenolic portion of the molecule, and its redox activity does not appear to be sensitive to organic bases which may be present in the cell. The electrochemical test indicating effective cytotoxicity with ferrocenylethylene phenol type moieties **4** and **5** is in agreement with the observation that only a ferrocenyl group in electronic communication with the phenolic group on this type of skeleton can produce an antiproliferative effect, in the submicromolar range, on breast cancer cell lines.

Modeling studies

We attempted to refine our results by molecular modeling of the compounds in the estrogen receptor site. The structure used is that of the α form of the human receptor (hERα) occupied by estradiol.[3,24] Only the amino acids that constitute the wall of the cavity have been retained. The estradiol was removed from the cavity and replaced successively with the different bioligands. All the heavy atoms of the amino acids of the cavity wall were then immobilized and the side chain of His524, as well as Met421, was liberated for compound **8**, since it is bulkier in this region. This was justified by the observation that this part of the cavity is flexible.[25] Energy minimization was then carried out using the Merck molecular force

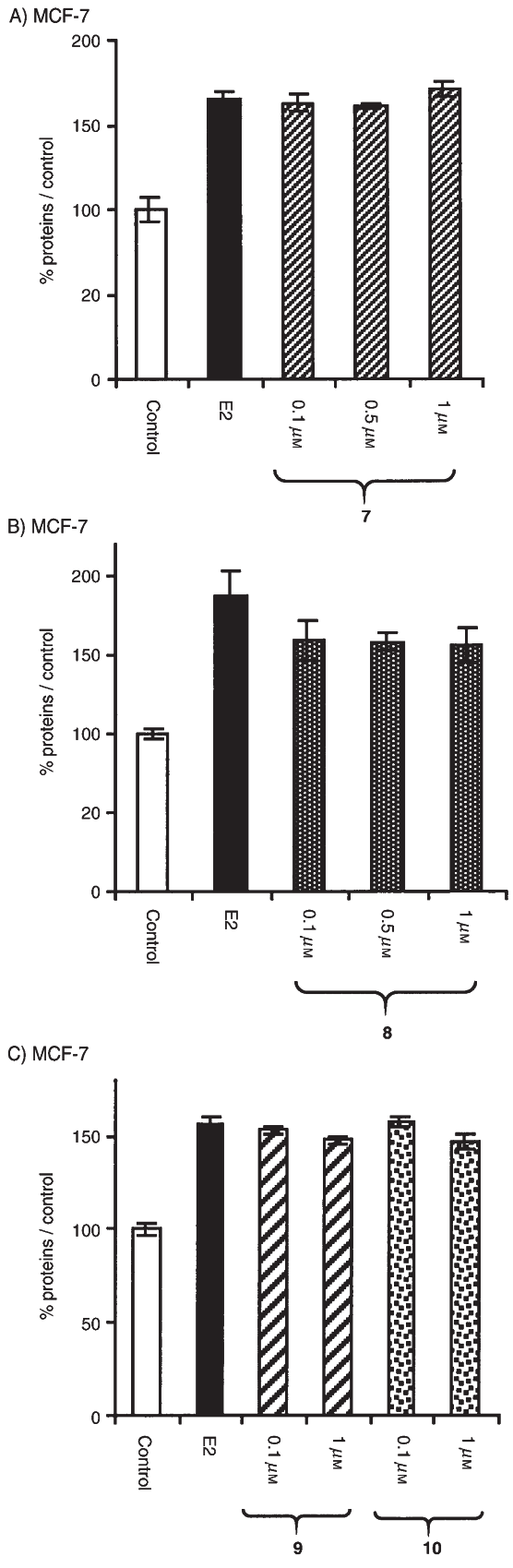


Figure 1. Study of the effect of estradiol (E_2 at 1 nM) and of the estradiol derivatives **7**, **8**, **9**, and **10** on the proliferation of MCF-7 cells (hormone-dependent breast cancer cells) after 5 days of culture. Representative data of one experiment performed twice with similar results (eight measurements ± limits of confidence; $P = 0.1$, $t = 1.895$).

field (MMFF). This allowed the ideal positions of the bioligands to be determined. Quantum mechanical semi-empirical PM3 methods were then used to determine the affinity of the bioligands for the cavity. This requires calculation of the energies of the bioligand-cavity group, of the cavity itself, and of the ligand, the latter two in the conformations they had in the molecular assemblies, to give the ΔrH° enthalpy variations of the reactions: bioligand + cavity → molecular assembly (Table 3). The bioligands are shown as compact models, with van der Waals spheres, the amino acids of the cavity wall are shown as sticks. The notable amino acids are labeled (Figure 2)

Table 3. Molecular modeling results of the compounds docked in the α form of the human estrogen receptor (hERα).

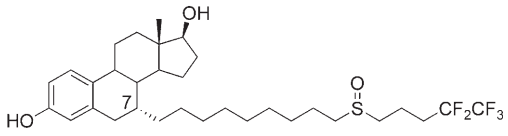
Compound	Volume [Å^3]	ΔrH° [kcal mol^{-1}]
17β-estradiol (E_2)	275	−27.5
7	487	−9.7
8	519	−23.3
9	294	−24.5
10	450	−9.8

Compound **9** with the SH group in position 7α binds in the ligand binding domain in a similar way to estradiol. The volume of **9** is slightly greater than that of estradiol (294 Å^3 for **9** versus 275 Å^3 for E_2) and the ΔrH° value indicates good interaction with the cavity, although less than for estradiol ($\Delta rH^\circ = -27.5$ kcal mol^{-1}). The various binding possibilities for **9** with Glu 353, Arg 394, and His 524 are standard. In the case of **10**, however, the presence of the -CH_2-ferrocenyl group on the thiol in position 7α produces an unusual binding mode.[26] In fact with **10** we have a 180° horizontal flip in the binding site so that the bulky -CH_2-ferrocenyl takes up the position normally occupied by the substituents at position 11β. Molecule **10** occupies a volume of 450 Å^3 and its binding is weakened ($\Delta rH^\circ = -9.8$ kcal mol^{-1}). It should be noted that the carbon chain is too short in this case to be able to generate an antiestrogenic effect similar to that provided by real antiestrogens such as ICI 182 780 and RU 58 668.

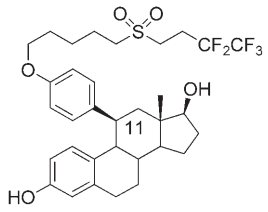
Compound **7**, with a ferrocenyl group bound to the carbon 17 of the steroid skeleton, shows steric constraint at this level in binding with the receptor ($\Delta rH^\circ = -9.7$ kcal mol^{-1}). This constraint is reduced for compound **8** which, although it is bulkier (519 Å^3 versus 487 Å^3), has the advantage of a more distant ferrocenyl group, thanks to the rigid acetylenic entity which is able to push aside the methionine 421 to give a ΔrH° of −23.3 kcal mol^{-1}. This modeling study allows visualization of the positioning of various steroids in the binding site of ERα. It is in agreement with the RBA values obtained for these compounds.

Conclusions

This work is part of ongoing research into the role and potential interest of organometallic species attached to a steroid skeleton or a SERM (selective estrogen receptor modula-



ICI 182 780



RU 58 668

tor).[27–30] Two main objectives have been sought in combining a delivery system that must retain its recognition for a given target, and an organometallic payload chosen for its biological potential. Firstly it was showed earlier that the ferrocenyl entity, which is stable in a nonoxidizing medium, and naturally lipophilic, could be oxidized to a ferrocenium and thus induce antiproliferative activity at high concentrations (0.01–0.1 mM) on cancer cell lines.[5,7] As the ferrocifen complexes are active in the submicromolar range we first decided to limit our studies on antiproliferative effects to concentrations equal to or less than one micromolar. In this case, the estrogenic effect of compounds **7**, **8**, and **10**, bearing a ferrocenic entity at positions 7α and 17α, is comparable to that of the natural bioligand. The observation of pure estrogenicity in an estrogenic molecule is not always the case. For example, complex **4** is both estrogenic and cytotoxic,[1] while some platinum complexes also show a combination of estrogenic and cytotoxic effects.[31,32]

In our case, we believe that this occurs when the easily oxidizable ferrocenyl antenna maintains an electronic connection with the phenol group, which activates it to produce a cytotoxic species of the quinone methide type **17.** The IC_{50} values obtained for **5** on hormone-dependent breast cancer cell lines such as MCF-7, and hormone-independent lines such as MDA-MB-231, are 0.7 and 0.6 μM respectively.[4] The importance of this unusual electronic connection in boosting antiproliferative effects thus becomes clear. In the absence of a specific pattern,

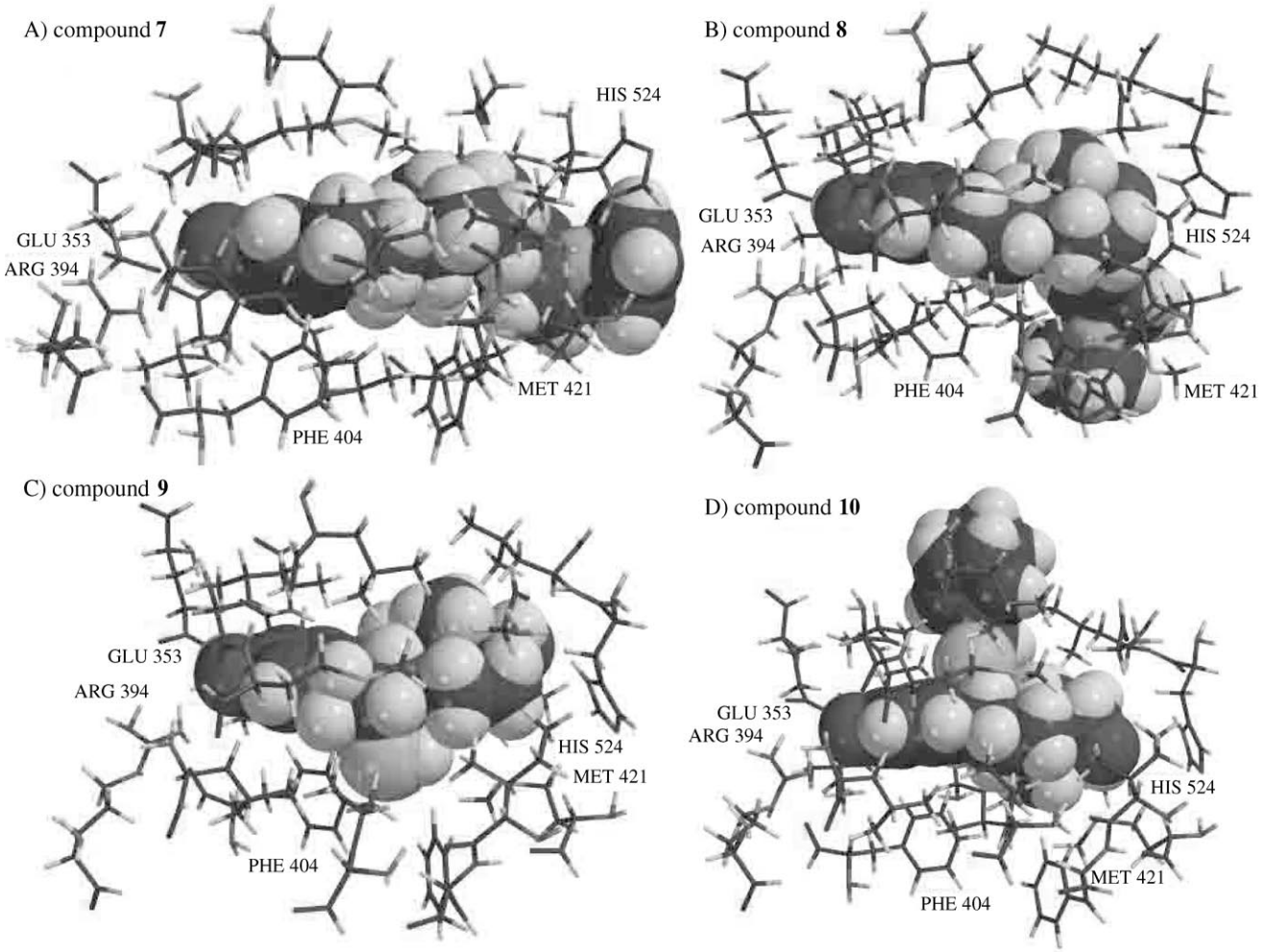


Figure 2. Molecular models for compounds **7**–**10.** The bioligands are represented as compact models of van der Waals sphere radii and the amino acids that constitute the wall of the cavity are shown as stick drawings. The amino acids, that interact with the bioligand are labeled.

there is no functional synergy as in **7**, **8**, and **10**, where the estrogenic activity of the vehicle is preserved. This does not mean that these species may not be interesting precursors for future studies in the area of radiopharmaceuticals. The use of other metals in place of the initial ferrocenyl to give complexes of $CpTc(CO)_3$ and $CpRe(CO)_3$ have already been reported.[19,33,34] These metallic elements give access to interesting radionuclides both for imaging (^{99m}Tc), and for radiotherapy (^{188}Re).[35] In terms of synthesis however there is still work to be done, and this will be the focus of future studies. Radiopharmaceutical molecules derived from **8** will be good synthetic targets in this regard.[36,37]

Experimental Section

General remarks

All reactions were performed under a dry argon atmosphere by using Schlenk techniques. The reagents and solvents were obtained from Aldrich Chemical Co and Janssen Chemical Co. Solvents were purified by conventional distillation techniques under argon. IR spectra were recorded on a Bomem Michelson 100 spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker AM200 spectrometer. Melting points were determined on a Kofler apparatus and are uncorrected. Mass spectra were obtained by the "Service de Spectometrie de Masse" of the ENSCP Paris.

Chemistry

*Synthesis of 7α-ferrocenylmethylthio-3,17β-dihidroxyestra-1,3,5(10)-triene (**10**):*

Trifluoroacetic acid (0.2 mL) was added to a solution of ferrocenylmethanol (1 mmol, 216 mg) prepared as described in ref. [14] and 7α-Mercapto-E_2 prepared as described in ref. [13] (1 mmol, 305 mg) in acetone (5 mL). After 15 h stirring at 25 °C, the reaction mixture was partitioned between water (10 mL) and Et_2O. The organic layer was washed with saturated aqueous $NaHCO_3$ (10 mL), then with water (10 mL), dried with $MgSO_4$, filtered and evaporated under reduced pressure. The pure product (294 mg, 58% yield) was obtained by chromatography on silica gel (pentane/ethyl acetate, 2:1), as a yellow fine powder; mp: 152 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 0.73$ (s, 3H, CH_3-18), 1.10–1.50 (m, 6H), 1.72 (dt, 1H, $J =$ 10.8 Hz and 2.6 Hz, H-8), 1.86 (dt, 1H, $J = 9.5$ Hz and 3.1 Hz, H-12), 2.06 (m, 1H, H-16), 2.30 (m, 1H, H-11), 2.40 (dt, 1H, H-9), 2.94 (d, 1H, $J = 16.6$ Hz, H-6), 3.03 (m, 1H, H-7), 3.15 (dd, 1H, $J = 16.6$ Hz and 4.3 Hz, H-6), 3.53 (s, 2H, SCH_2), 3.73 (t, 1H, $J = 8.5$ Hz, H-17), 4.11 (m, 3H, C_5H_4), 4.12 (s, 5H, C_5H_5), 4.20 (m, 1H, C_5H_4), 4.69 (s, 1H, OH), 6.55 (d, 1H, $J = 2.6$ Hz, H-4), 6.63 (dd, 1H, $J = 8.4$ Hz and 2.6 Hz, H-2), 7.14 ppm (d, 1H, $J = 8.4$ Hz, H-1); MS (FAB) 502 [M^+], 500 [$M-2$H]; MS (EI, HR): calcd [M]$^+$ 502.16289, found [M^+] 502.16261; anal. calcd for $C_{29}H_{34}FeO_2S,H_2O$: C 66.92, H 6.97, found: C 66.84, H 7.23.

Biochemical studies

New stock solutions of the organometallic complexes (1×10^{-3} M or 1×10^{-2} M) in ethanol or DMSO were prepared for each experiment. Serial dilutions in ethanol or DMSO were prepared just prior to use.

Determination of the relative binding affinity (RBA) of the compounds for the estrogen receptor α (ERα):

Lamb uteri from young animals (maximum weight 7 g) were obtained from the slaughterhouse of Mantes-la-Jolie, France. They were immediately frozen and kept frozen in liquid nitrogen prior to use. Lamb uterine cytosol was prepared as follows: a portion of 1 g of uterus was thawed, minced, and then homogenized with an Ultra-Turrax in 8 mL of buffer (0.05 M Tris-HCl, 0.25 M sucrose, 0.1% β-mercaptoethanol, pH 7.4 at 25 °C). The homogenate was centrifuged at 40000 t min^{-1} for 35 min in a Kontron TFT 65.13 rotor of a Kontron T1160. The volume of the cytosol was then adjusted to 2 mg mL^{-1} of proteins. Aliquots (200 μL) of the cytosol were incubated for 3 h at 0 °C with [6,7-^{3}H]-estradiol (2×10^{-9} M, specific activity 1.96 TBq mmol^{-1}) in the presence of nine concentrations of estradiol or of the hormones to be tested (ranging from 10^{-10} M to10^{-6} M; final volume of ethanol in the medium equal to 5%). At the end of the incubation period, the free and bound fractions of the tracer were separated by protamine sulfate precipitation as follows: 200 μL of buffer containing 1 mg/mL 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 fiber paper (Whatman GF/C) under a moderate vacuum and washed with 40 mL of ice-cold buffer. The filter papers were then transferred to scintillation vials and 5 mL of scintillation fluid (Amersham BCS scintillation cocktail) was added. Radioactivity was measured in a Packard IND1401 counter. The concentration of unlabeled steroid (IC_{50} value) required to displace 50% of the bound [^{3}H]-estradiol was then determined for each steroid tested. The relative binding affinity of each compound (RBA) is equal to the ratio: (IC_{50} of E_2/IC_{50} of the tested compound) 100. Consequently the RBA value of estradiol is by definition equal to 100%.

Log $P_{o/w}$ determinations:

Log $P_{o/w}$ values were estimated from values of log k'_w (k'_w = capacity ratio in the absence of methanol), determined by HPLC chromatography according to Minick[38] and Pomper[39] using a Macherey–Nagel EC250/3 Nucleosil 100–5C18HD column. The UV detector was set to 277 nm. The organic portion of the mobile phase was composed of methanol containing 0.25% (*v*/*v*) 1-octanol. The aqueous portion was an octanol-saturated buffer prepared from 0.02 M 3-morpholinepropanesulfonic acid (MOPS) and 0.15% (*v*/*v*) *n*-decylamine; pH was adjusted to 7.4 with aq. NaOH (0.1 M). The flow rate was 0.45 mL min^{-1}. The steroids were dissolved at 1 mM concentration in ethanol and 20 μL was injected. Column void volume was estimated from retention time of uracil (t_0), which was included as an unretained internal reference with each run. Values of k' were obtained from the steroids retention time (t_R) according to $k' = (t_R - t_0)/t_0$. The log k'_w was determined by linear extrapolation of log k' versus ϕ methanol (ϕ = volume fraction of methanol); data acquired with $0.55 \leq \phi \leq 0.85$.

Cell culture

Culture materials:

Dulbecco's modified eagle medium (DMEM) with phenol red/Glutamax I, Dulbecco's modified eagle medium (DMEM) without phenol red, Glutamax I, and fetal bovine serum (FBS) were purchased from Gibco; estradiol from Sigma. MCF-7 and MDA-MB-231 cells were obtained from the Human Tumor Cell Bank.

Culture conditions:

Cells were maintained in a monolayer culture in DMEM with phenol red/Glutamax I (Gibco) supplemented with 9% decomplemented fetal bovine serum and 0.9% of kanamycin at 37 °C in a 5% CO_2/air humidified incubator. For proliferation assays, cells were seeded in 24-well sterile plates. MCF-7 cells were plated in 1 mL in DMEM with phenol red/Glutamax I, supplemented with 9% decomplemented and hormone-depleted fetal bovine serum and 0.9% kanamycin at a density of 25000 to 35000 cells per mL; MDA-MB-231 cells were plated in 1 mL of DMEM without phenol red, supplemented with 9% decomplemented and hormone-depleted fetal bovine serum, 0.9% kanamycin, 0.9% Glutamax I at a

density of 15000 to 25000 cells per mL and incubated 24 h. The following day (D_0), 1 mL of the same medium containing the compounds to be tested was added to the plates (final concentration of ethanol or DMSO = 0.5%, 4 wells per concentration). After 3 days (D3) the incubation medium was removed and 2 mL of fresh medium containing the compounds was added. After 5 or 6 days (D_5 for **7** and **8** and D_6 for **9** and **10**) the total protein content of the wells was quantified by coloration with methylene blue. One mL of a methylene blue solution (1 mg mL^{-1} in a 50:50 water/MeOH mixture) was added to each well and incubated 1 h at room temperature. The solution was removed and the plate was gently washed with water. Two mL of HCl 0.1 M was then added to each well and the plate was incubated for 1 h at 37 °C. Then 100 μL of each well was transferred to 96-well plates (two separate measurements for each wells) and the absorbance of each well was measured at 655 nm with a Bio-Rad 550 microplate reader. The results are expressed as the percentage of proteins versus the control. IC_{50} values (concentration of the compound that produces 50% decrease of the protein content) were calculated from curves constructed by plotting % proteins (%) versus drug concentration. Experiments were performed in duplicate.

Electrochemical studies

Cyclic voltammograms were obtained using an Autolab PGStat potentiostat, driven by GPES software, (General Purpose Electrochemical System, Version 4.8, EcoChemie B.V., Utrecht, the Netherlands) a platinum wire counterelectrode, a 500 μm platinum disc working electrode, and an aqueous standard calomel reference electrode. Analyte solutions were 1–2 mM in wet, oxygenated MeOH with 0.1 M Bu_4NBF_4 supporting electrolyte.

Modeling studies

Molecular modeling studies were carried out using Mac Spartam Pro.[40]

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