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Synthesis and characterisation of estrogenic carriers for cytotoxic Pt(II) fragments: biological activity of the resulting complexes†

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This paper describes the synthesis and the spectroscopic characterisation of *cis*-dichloro[*N*-(4-(17α-ethynylestradiolyl)-benzyl)-ethylenediamine]platinum(II) and *cis*-diamino[2-(4-(17α-ethynylestradiolyl)-benzoylamino)-malonato]platinum(II). These complexes were synthesised in good yield according to multi-step procedures based on the classical and non-classical Sonogashira coupling reaction, respectively. These compounds retain an acceptable degree of relative binding affinity (RBA) for the α form of estrogen receptor. Combined treatment of breast cancer cell lines, namely hormone-sensitive MCF-7 and hormone-insensitive MDA-MB-231 cell lines, indicates that these compounds maintain agonistic activity so that the potential advantage in vehiculation of the cytotoxic moiety by means of the receptor system is counteracted by the proliferative effect of the estrogenic component of the entire molecule, especially at low concentrations.

Introduction

Cisplatin and its second-generation analogues, namely carboplatin and oxaliplatin, have afforded good results in the treatment of solid tumours, especially testicular, ovarian and colon cancer (oxaliplatin only). However, there are several major drawbacks to their clinical use, such as poor selectivity between malignant and normal cells (resulting in severe side effects), and the tendency to provoke chemoresistance. The strategy of drug targeting and delivery could be applied to tumours that exhibit biochemical differences from normal tissue. Tumours possessing hormone receptors represent a large class of malignancies that includes breast, endometrium and prostate cancers. Given that 60–70% of mammary carcinomas express estrogen receptors, at least at the early stage, an estrogen-like ligand might prove useful in delivering cytotoxic agents, in particular the cisplatin [Pt(II)X_2] fragment (X = leaving group: halide or carboxylate), to such estrogen-responsive (ER+) neoplasms.[1–4] Provided the relative binding affinity (RBA) for the estrogen receptor of the resulting Pt(II)-estrogen complexes is high enough, the receptor system would selectively recognise it. Furthermore, if the cytotoxic complexes exhibit agonistic activity, the receptor would vehiculate and approximate them to key sequences of genomes in mammary tumour cells.[1,5] This would require a coordinating arm capable of linking the PtX_2-unit to the estrogen carrier. In principle, a suitable arm would consist of a rigid spacer ending in a diamine functionality (ethylenediamine, piperazine, *etc.*). In order to link the estrogen to the arm, several reactions are available, but they often require that the $-NH_2$ groups be protected (*e.g.* with *tert*-butyl carbonate, Boc). Thus, the procedure involves multi-step reactions, which generally afford low yields. The use of tertiary amines is therefore generally preferred, since it does not call for protection or deprotection steps. However, the substitution in the amine carrier groups diminishes the cytotoxic activity of the *cis*-Pt(II)X_2-moiety.[6] Moreover, after hydrolysis of the PtX_2-unit, the necessary reaction between the resulting estradiol–arm–Pt(II) nucleophile and DNA can be obstructed by the steric encumbrance.[7]

Alternatively, a $[Pt(NH_3)_2]^{2+}$ moiety could be delivered to DNA by forming the same *cis*-Pt$(NH_3)_2$d(GpG) intrastrand adduct that cisplatin does. This can be achieved by tethering the $[Pt(NH_3)_2]^{2+}$ moiety to a coordinating arm ending in a leaving group, such as the malonate group.[8] It has been reported that dicarboxylate ligands strongly increase the solubility of the corresponding complexes.[9] We are aware that the hydrolysis of malonato complexes at physiological pH is enormously slower *in vitro* than that of corresponding chloride complexes, however, enzymatic removal seems to be very efficient *in vivo*.[10–12]

17α-Ethynylestradiol represents a good starting molecule by virtue of its reactive acetylene hydrogen atom, which can easily be linked to arms ending with either a diamine or a dicarboxylate group. Considerable effort has been devoted to optimizing the reactions of organic frameworks bearing alkynyl moieties. Among these, we have chosen classical Sonogashira[13] and non-classical Sonogashira[14] metal-catalysed carbon–carbon bond-forming reactions with terminal alkynes and aryl halides.

In this paper we report on the synthesis, characterisation and biological activity of two ethynylestradiol-ligands and their corresponding Pt(II)-complexes (Fig. 1).

Results and discussion

Ligand synthesis

Synthesis of *N*-[4-(17α-ethynylestradiolyl)-benzyl]-ethylenediamine, 4. Originally introduced by Inhoffen and Hohlweg in 1938,[15] the majority of the synthetic approaches to ethynylestradiol analogues have relied on the addition of acetylide to the resident ketone at C-17 in estrone. From a retrosynthetic perspective, we envisioned installation of the five-carbon spacer late in the synthesis (Scheme 1), thereby permitting construction of ligand **4** from the precursor 17α-ethynylestradiol. Accordingly, assembly of the requisite five-carbon linker would entail coupling of *N*-(4-iodobenzyl)-*N*,*N*′-diBoc-ethylenediamine (**2**) with 17α-ethynylestradiol, exploiting a Cassar–Heck–Sonogashira C(sp^2)–C(sp) cross-coupling reaction.[16–18] Our starting point

† Electronic supplementary information (ESI) available: NMR spectra for ligands **4** and **6** and complexes **7** and **8**. See http://dx.doi.org/10.1039/b507716h

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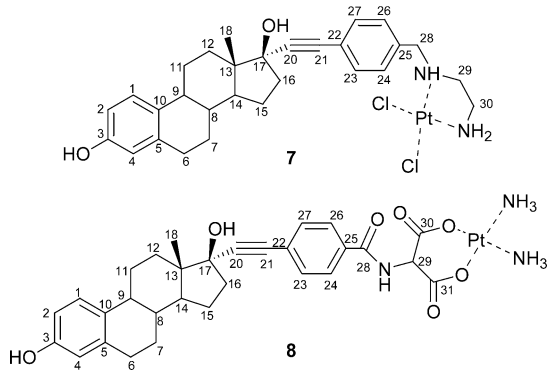
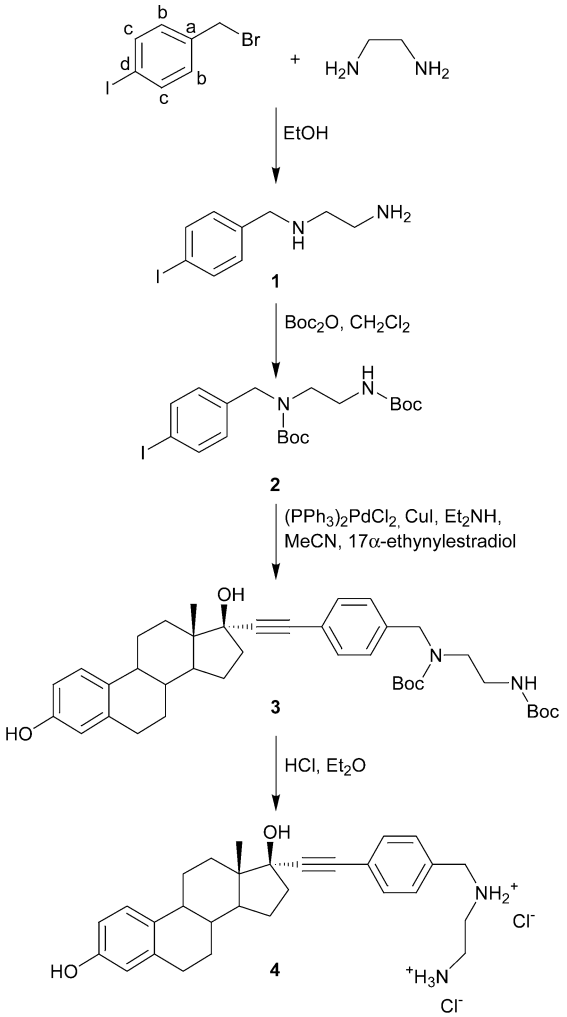


Fig. 1 Sketch of structures of the platinum complexes *cis*-dichloro[*N*-(4-(17α-ethynylestradiolyl)-benzyl)-ethylenediamine]platinum(II), **7**, and *cis*-diamino[2-(4-(17α-ethynylestradiolyl)-benzoylamino)-malonato]-platinum(II), **8**.

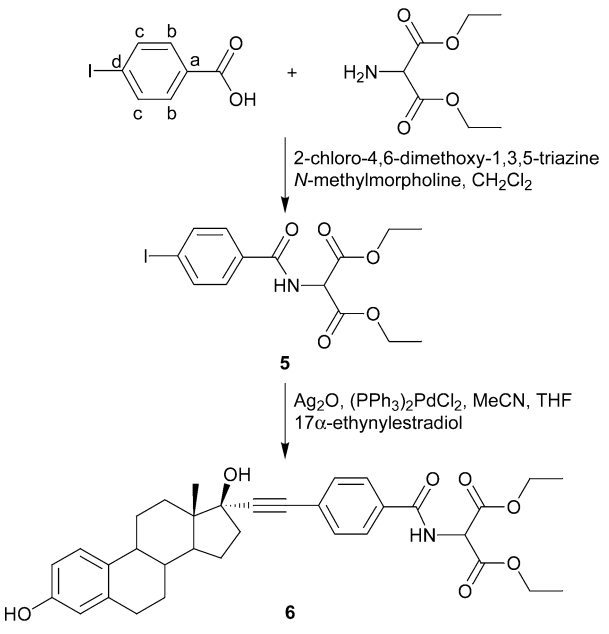


Scheme 1 Reaction pathway for the synthesis of *N*-[4-(17α-ethynylestradiolyl)-benzyl]-ethylenediamine, **4**.

for **4** was the commercially available 4-iodobenzyl bromide. This reacted with excess ethylenediamine[19] in EtOH to provide the corresponding *N*-(4-iodobenzyl)-ethylenediamine, which, by protection with di-*tert*-butyl dicarbonate (Boc_2O) in CH_2Cl_2, afforded **2**. Sonogashira reaction of **2** with 17α-ethynylestradiol in the presence of $(PPh_3)_2PdCl_2$, CuI, Et_2NH in MeCN gave *N*-(4-(17α-ethynylestradiolyl)-benzyl)-*N*,*N*′-diBoc-ethylenediamine (**3**), and subsequent removal of the Boc groups (HCl in Et_2O) gave the required internal alkyne **4** as a dihydrochloride with an overall yield of 42% (Scheme 1).

Protection of the amino groups in *N*-(4-iodobenzyl)-ethylenediamine was imperative for the alkyne coupling reaction in order to avoid competing *ortho*-palladation of the substrate.[20]

Synthesis of 2-[4-(17α-ethynylestradiolyl)-benzoylamino]-malonic acid diethyl ester, 6. The steroid-malonate ester **6** was prepared starting from commercially available diethyl 2-aminomalonate hydrochloride. Regarding the connecting arm between the 17α-ethynylestradiol moiety and aminomalonate, we chose to insert a rigid, suitably functionalised, aromatic structure. 4-Iodobenzoic acid proved to be a good starting material. The first step was the synthesis of a peptide bond between aminomalonate and 4-iodobenzoic acid. Although it is generally accepted that carbodiimides are the most efficient condensing agents, the formation of by-products prompted us to choose 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT) as a carboxylic acid activator.[21] The best result in the activation stage was achieved by adding *N*-methylmorpholine dropwise to a solution of diethyl 2-aminomalonate and 4-iodobenzoic acid in CH_2Cl_2 at −5 °C. The pure 2-(4-iodobenzoylamino)-malonic acid diethyl ester **5** was obtained after recrystallization with a yield of 71.5%. The following synthetic step was meant to have been a Sonogashira cross-coupling reaction between the intermediate **5** and 17α-ethynylestradiol. Unfortunately, under classical Sonogashira reaction conditions (CuI, alkylamine as the solvent or in stoichiometric amounts, and Pd-complexes) only a trace amount of the expected product was recovered. The literature[22] indicates that copper(I) iodide as co-catalyst in the presence of oxygen induces a homocoupling reaction (Glaser-type reaction)[23] of terminal alkynes; moreover, the presence of the amine may cause deprotonation of the malonate acidic proton and generation of palladium enolate species.[24] Copper-free methodologies employing amines as solvents have been described.[25] The first described copper- and amine-free procedure involves the use of stoichiometric amounts of silver(I) oxide for aryl iodide.[14] In a typical procedure, malonate ester **5** (1.5 mmol) and 17α-ethynylestradiol (1.5 mmol) were dissolved in THF (15 mL). $(PPh_3)_2PdCl_2$ (5%) and Ag_2O (1.5 mmol) were added, and the reaction mixture was warmed to 60 °C. Routine workup and purification of the crude mixture by column chromatography afforded the expected coupling product **6** with a yield of 61.1% (Scheme 2).



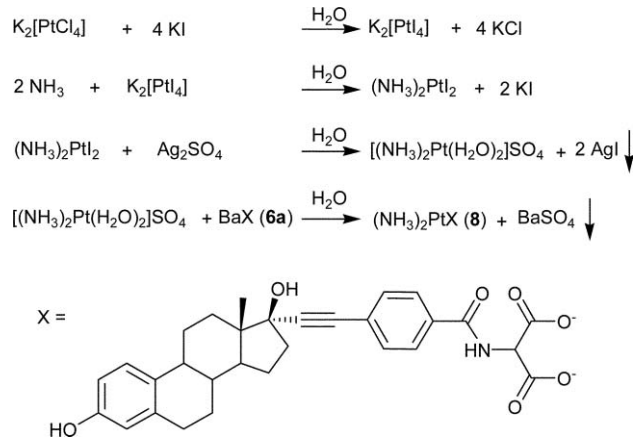
Scheme 2 Reaction pathway for the synthesis of 2-[4-(17α-ethynylestradiolyl)-benzoylamino]-malonic acid diethyl ester, **6**.

Platinum coordination

Synthesis of *cis*-dichloro[*N*-(4-(17α-ethynylestradiolyl)-benzyl)-ethylenediamine]platinum(II), 7. Compound **7** was obtained in good yield by reaction of $K_2[PtCl_4]$ and **4** according to Miller's procedure for platinum coordination.[26]

$K_2[PtCl_4]$ was added to an aqueous solution of *N*-(4-(17α-ethynylestradiolyl)-benzyl)-ethylenediamine dihydrochloride at about 60 °C. During the reaction, the pH was kept at *ca.* 6.0 by the dropwise addition of 0.1 N NaOH. The pH value is important at this stage of the reaction: it must be high enough to deprotonate the diamine ligand, thus making it suitable for platinum coordination, but not so high as to cause the formation of inactive hydroxo-Pt species.

Synthesis of *cis*-diamino[2-(4-(17α-ethynylestradiolyl)-benzoylamino)-malonato]platinum(II), 8. The synthesis of the hormone–malonato platinum(II) complex **8** is shown in Scheme 3. The complex was prepared by reacting NH_3 with $K_2[PtI_4]$, generated *in situ* according to Dhara's method,[27,28] after which iodide ions were replaced with water by means of Ag_2SO_4. The treatment of the estradiol-substituted malonic ester **6** with aqueous barium hydroxide gave the corresponding barium salt (**6a**). The reaction of this barium salt with an aqueous solution of *cis*-diaminodiaquoplatinum(II) sulfate yielded the final complex **8**.[29]



Scheme 3 Platinum coordination of 2-(4-(17α-ethynylestradiolyl)-benzoylamino)-malonato barium salt (**6a**).

Spectroscopic characterisation

The molecular formula of each compound was verified by comparing the molecular ion peak in the DCI or ESI mass spectrum with the correct isotope distribution. The ESI-MS spectrum of carboxylato complex **8**, run in water–methanol (1 : 1), showed the molecular ion peak $[M + H]^+$. The dichloride complex **7** featured an ESI-MS spectrum in water–DMSO (3 : 1) whose main signal was attributable to the $[M–Cl + DMSO]^+$ ion.[30]

As a general trend, the ^1H and ^{13}C chemical shifts in the complexes were shifted to lower field when compared to free ligands, and atoms closer to the Pt centre were more strongly affected. 18-CH_3 appeared between 0.4 and 0.9 ppm in the ^1H NMR spectra of the complexes as well as of the parent hormones. The aliphatic skeleton protons were spread between 1.0 and 2.8 ppm, whereas the aromatic protons 1-H, 2-H and 4-H formed an AMX pattern ($^3J_{1,2}$ 8.4, $^4J_{2,4}$ 2.6 Hz) between 8.0 and 6.1 ppm and the aromatic protons of the linking arm appeared between 7.6 and 7.2 ppm.[31,32]

In the ^{13}C spectra the aliphatic carbons were spread between 12 and 52 ppm; whereas aromatic carbons appeared between 112 and 157 ppm. The signals of C-20, C-21 and C-17 appeared from 98 to 74 ppm. The assignment of resonances due to C-8 and C-12 in $(CD_3)_2SO$ was not possible owing to their partial overlap with the carbon signals of the solvents.

The purity of the complexes was further verified by ^{195}Pt NMR spectroscopy. Since the δ(Pt) chemical shift depends on the electronic density on the Pt atom, it is sensitive to the nature of the bound donor atoms and each species will show a different signal.

Since complex **7** was not soluble in water, the spectrum was measured in $(CD_3)_2SO$. The only signal detectable after accumulation was observed at −3307 ppm; this signal was attributable to the chloro(dimethylsulfoxide)[*N*-(4-(17α-ethynylestradiolyl)-benzyl)-ethylenediamine]platinum(II) species. This substitution of DMSO for chloride in **7** is in tune with MS results, and is typical of all $Pt(II)Cl_2$ moieties.[30]

As far as **8** is concerned, as previously described by Gibson *et al.*,[33] the presence of the nitrogen atom in the α position relative to the malonato unit promotes a coordination isomerism of the (O,O′)-chelate **8** to the (N,O)-chelate **8** (see Fig. 2). Indeed, Gibson described the reaction between *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ and 2-aminomalonate in water and showed that the kinetic product (O,O′)-chelate was first formed and then quickly isomerised to the thermodynamically stable (N,O)-chelate. Complex **8** is barely soluble in water: when its ^{195}Pt spectrum was run in $(CD_3)_2SO$ at room temperature, it exhibited only one signal at −1718 ppm. The spectrum in CD_3OD at rt shows two peaks at −1737 ppm and −2171 ppm attributable to the (O,O′)-chelate **8** and the (N,O)-chelate **8**, respectively, in a 6 : 1 ratio. With the hormonal ligand, the conversion is slower because the nitrogen responsible for this isomerisation is less suitable as an amidic nitrogen for platinum coordination than the amine one. The steric hindrance further inhibits the isomerisation. Thus, the ratio between the (O,O′)-chelate **8** and the (N,O)-chelate **8** is still in favour of the former.

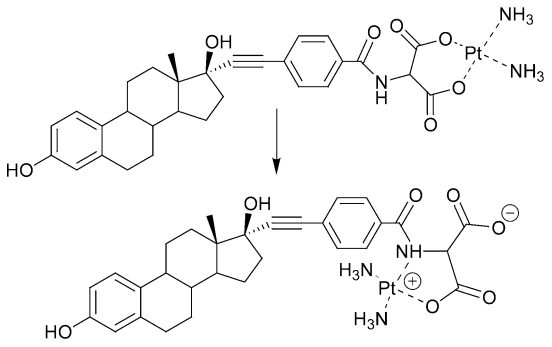


Fig. 2 Coordination isomers of **8**.

A similar behaviour has also been reported for some macromolecular platinum complexes having the same coordination geometry.[34,35]

This kind of isomerisation is solvent-dependent. According to the data reported by Lee *et al.*,[36] the coordination mode of the anionic ligand depends on the hydrogen bonding ability of the solvent. Since the (N,O)-chelate has a zwitterionic form, the hydrogen bonds between protic solvent molecules and the zwitterionic dipole seem to be responsible for its stabilisation. Methanol has a hydrogen bonding ability that allows the two chelation modes to coexist at room temperature. The above (N,O)-chelate is a triamine cationic platinum complex usually considered inactive as an antitumour agent. Hollis *et al.*,[37] however, showed a non negligible antitumour activity for triamine cationic Pt(II) complexes.

Structure optimization by molecular mechanics (MM)

Although we have not obtained X-ray quality crystals of **7** and **8**, a reasonable model of their structures was obtained using the HyperChem™ Package. It was constructed by exploiting the published X-ray structures of the steroidal skeleton and of platinum(II) complexes having the same coordination sphere.

The starting structure of **7** was constructed by replacing the 17α proton of 17β-estradiol[38] with the –C≡C-*p*Ph–CH_2-spacer, whose bond distances and angles were adjusted to typical values.

The structure of the coordination sphere was derived from that of *cis*-[dichlorideethylenediamineplatinum(II)],[39] replacing one of the $-NH_2$ protons with the methylene group of the spacer. The resulting structure was energy-minimized in the Amber force field using a steepest descent algorithm, leaving the platinum coordination geometry unchanged.

The starting point for the structure of (O,O′)-chelate **8** was the same. For the platinum-containing moiety the structure of *cis*-[diaminomalonatoplatinum(II)][40] was chosen as a model. One of the $-CH_2$ protons was replaced with the amidic group of the –C≡C-*p*Ph–CO–NH-spacer. After adjusting bond distances and angles to the normal value, the resulting structure was energy-minimized as described above.

Although this procedure is rather approximate, given that the metal centre is a fixed unit, the method provides a reasonable model of the overall molecular geometry. The resulting structures are shown in Fig. 3.

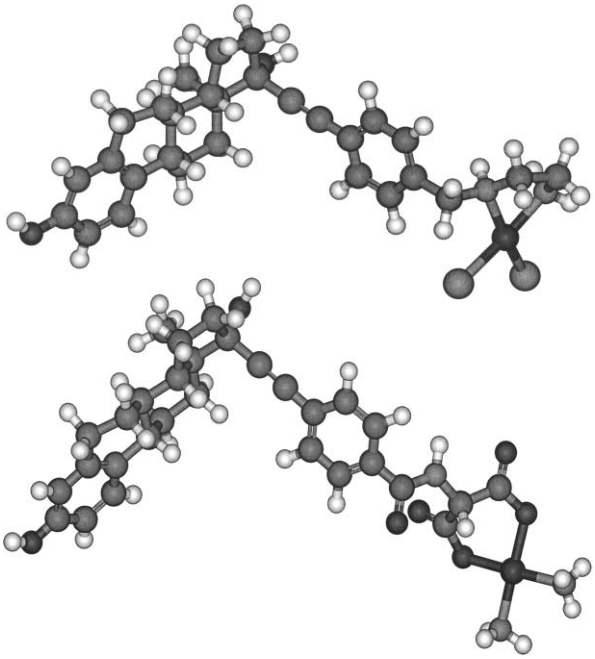


Fig. 3 Optimized structures of **7** and **8**.

As immediately visible in the optimized structures, the rigid spacer keeps the platinum core well away from the two –OH groups responsible for binding to the receptors. Interestingly enough, the MM model of the (N,O)-chelate **8** again shows that the Pt unit is still far away from the estrogen domain.

Biochemical and biological results

We have measured the relative binding affinity (RBA) of estradiol bio-ligands **4** and **6** for the α form of the estrogen receptor (ERα), which indicates whether or not these compounds could still be recognized by this receptor (these measurements were performed in a competitive radioreceptor binding assay using lamb uterine cytosol as the source of ERα and [^{3}H]-estradiol as a tracer) (Table 1). We have also determined the lipophilicity of **4** and **6** (octanol/water partition coefficient, log $P_{o/w}$: this value, which is determined by means of HPLC, is a rough measure of the drug lipophilicity, related to its ability to cross cell membranes) (Table 1).

Finally, we have studied the effects of the bio-ligands **4** and **6**, and the corresponding Pt(II) complexes **7** and **8**, on the proliferation of a human mammary adenocarcinoma cell line (MCF-7), that expresses estrogen receptor (ER+) and responds to estrogen activity. As control for estrogen activity we have employed a hormone-insensitive human mammary MDA-MB-231 cell line (Figs. 4 and 5).

Table 1 RBA and log $P_{o/w}$ values

Compounds	RBA (%)[a] (ERα)	Log $P_{o/w}$[a]
17β-Estradiol	100[b]	3.14 ± 0.07
17α-Ethynylestradiol	70[c]	3.25 ± 0.09
4	1.0 ± 0.1	2.21 ± 0.04
6	5.1 ± 0.4	4.97 ± 0.08

[a] The experimental values are means ± SD of 3 independent experiments. [b] By definition. [c] Ref. 41

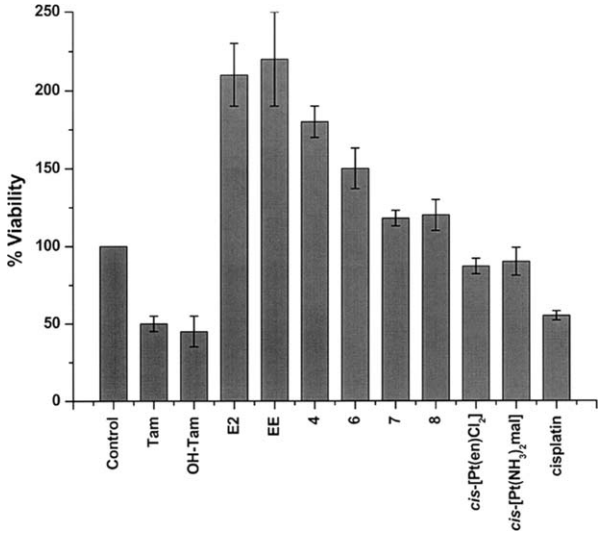


Fig. 4 Effect on MCF-7 cell viability. Cells were challenged with compounds under study at 1 μM concentration for 5 day continuous treatment.

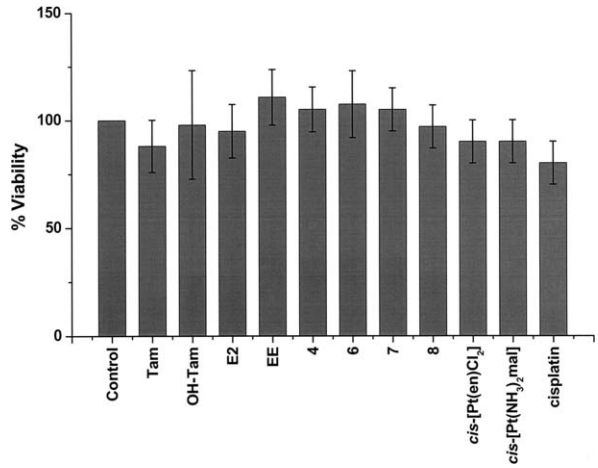


Fig. 5 Effect on MDA-MB-231 cell viability. Cells were challenged with compounds under study at 1 μM concentration for 5 day continuous treatment.

The RBA values indicate that compounds **4** and **6** do interact with the receptor, although their affinity is moderate as compared to 17α-ethynylestradiol, thus suggesting that they may behave as carriers for Pt delivery to the nucleus.

The RBA value for **4** was somewhat disappointing (only 1%). However, this result finds an explanation in the modelling study by Foy *et al.*[42] on the estrogen receptor binding site. This study considered the affinity of a series of 17α-arylestradiol derivatives, demonstrating that only the *p*-NH_2 substituent dramatically reduced the RBA value. The protonated amino hydrophilic group underwent an electrostatic repulsion, likely due to a methionine group, in the active site of the estrogen receptor.

We have recently shown for a similar series of 17α-ethynylestradiol-carriers ending in ethylenediamine or piperazine, that their coordination by a PtX_2-moiety, which engages the lone pair present on nitrogen ligands, thus preventing their protonation, leads to a significant increase in the RBA.[43] On the contrary, the replacement for **8** of two Et groups with the $[Pt(NH_3)_2]^{2+}$ moiety should lead to a drop in RBA.

In this context, an estimated RBA value of both compounds **7** and **8** is about 2%, in the same order of magnitude as the potent selective estrogen receptor modulator (SERM) tamoxifen (Tam).[44] Thus, we have indirectly evaluated the affinity of complexes **7** and **8** for the receptor on the basis of their proliferation effect on ER+ and ER- cell lines, along with that of the corresponding ligands and reference compounds. Interestingly enough, the estimated RBA values as well as the effect on cell growth are higher for complexes **7** and **8** with respect to those of previously reported 17α-ethynylestradiol-ethylenediamine-Pt(II)-malonato complexes.[43] The presence of an aromatic spacer in the arm between estradiol and ethylenediamine moieties improves their biological activity. This is in tune with previous studies from Bérubé *et al.*[45–47] on triphenylethylene- and estradiol-Pt(II) complexes having 6–10 aliphatic carbon atom spacers.

The effect of compounds **4–8** on MCF-7 growth was compared to that of 17β-estradiol (E2) and 17α-ethynylestradiol (EE), the reference estrogens, tamoxifen (Tam) and hydroxytamoxifen (OH–Tam), two SERMs, *cis*-$[Pt(en)Cl_2]$ (en = ethylenediamine) and *cis*-$[Pt(NH_3)_2mal]$ (mal = malonato), the model complexes, and cisplatin as a reference antiproliferative agent, all at a concentration of 1 μM (Fig. 4). The bars in the histogram show the inhibitory (for Tam and OH–Tam) or proliferative (for E2, EE, **4** and **6**) effect of the compounds relative to the control.

As expected, all the compounds under study, except those containing the cytotoxic Pt(II) moieties, have no significant effect on the MDA-MB-231 cell line (Fig. 5). The proliferative effect observed on MCF-7 cells is therefore clearly a hormonal effect mediated by the estrogen receptor.[48]

As far as non-hormonal Pt(II) complexes are concerned, the cytotoxic effect of *cis*-$[Pt(en)Cl_2]$, *cis*-$[Pt(NH_3)_2mal]$, and cisplatin is lower for MDA-MB-231 with respect to that of the MCF-7 cell line, as previously reported in the literature.[49]

Returning to the question of viability of the MCF-7 cell line, it is disappointing that complexes **7** and **8** showed no advantage in terms of inhibition of proliferation over the corresponding model complexes *cis*-$[Pt(en)Cl_2]$, *cis*-$[Pt(NH_3)_2mal]$ or cisplatin itself. This failure might be attributable to several factors: *i*) the decrease in cytotoxicity due to the steric effect of chelating arms necessary for linking the Pt(II)-moiety to the estrogen;[6] *ii*) the isomerisation from (O,O′) to (N,O)-chelate forms in **8**; *iii*) the limited number of receptors able to act as a shuttle for the estrogen–platinum intracellular adduct (see bio-stoichiometry in the experimental section) and *iv*) most importantly, the estrogenic (agonistic) activity of the overall assembly. Indeed comparison of the effects of the panel of compounds on ER+ cells clearly indicates that complexes **7** and **8** are recognised by the receptor as well as the ligands **4** and **6**, and all of them produce an overall proliferative effect. This is because the estrogenic effect generally operates at a nanomolar level,[50] whereas a classical cytotoxic effect for cisplatin-like complexes is usually comprised in the range 1–10 μM.

Indeed, at 0.1 μM concentration the hormonal Pt(II) complexes, namely **7** and **8** had an overall proliferative effect (Fig. 6).

In a hypothetical *in vivo* model, it is reasonable to suppose that, due to pharmacokinetic reasons, the cancer cells are exposed to optimal cytotoxic levels of these compounds only for short periods, whereas, until the plasma concentration drops to 0, the stimulatory effects of the compounds (due to lower concentration) will be prevalent.

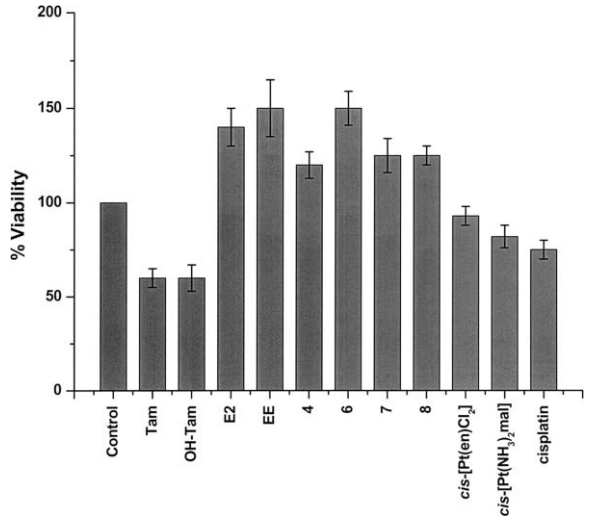


Fig. 6 Effect on MCF-7 cell viability. Cells were challenged with compounds under study at 0.1 μM concentration for 5 day continuous treatment.

We treated both cell lines with a mixture at equimolar concentrations of estradiol (the natural ligand for ERα+) and cisplatin, the most active platinum drug. The data obtained from this combination indicate that the cytotoxic effect of cisplatin is overcome by the growth stimulation of estradiol at concentrations lower than 10^{-6} M (Fig. 7). We are aware that the two compounds probably differ in pharmacokinetics and cellular uptake, but the gross effect appears nonetheless to be incontrovertible. It is important to recall that the treatment of cells with the mixture of both compounds at 10^{-5} M (data not shown) had a negative affect on cell viability (all the cells died within 5 days). While this concentration corresponds to the optimal activity for cisplatin, the same concentration represents an unphysiological condition for E2, leading to strong interference of growth.[50]

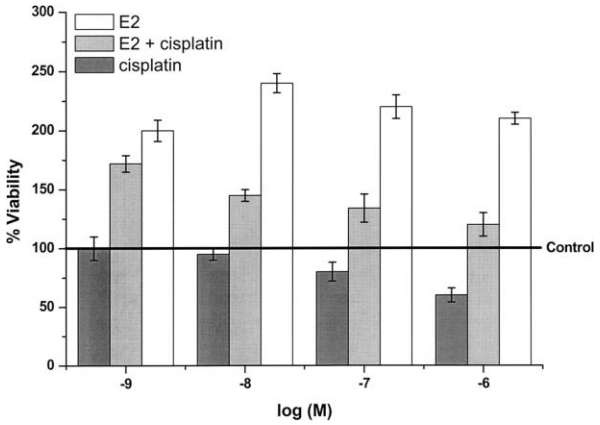


Fig. 7 Effect of the equimolar combination of E2 and cisplatin on MCF-7 cell viability (5 day continuous treatment).

Seminal investigations on the action of both steroidal hormones and cisplatin on breast cancer cells have been made by Lippard *et al.*[51–53] The aim of these investigations, as well as the concentrations and treatment times involved, were profoundly different from those of the present study.

Short exposure periods of MCF-7 cells to low concentrations of E2 (*i.e.* 10^{-7} M for 4 h) were shown to induce high mobility group (HMG) protein overexpression and, hence, protection of the cisplatin-DNA adduct by the nucleotide excision repair (NER) system. In this short time span, it was impossible for E2 to induce significant growth stimulation, and the overall result was an increased sensitivity of the hormone-treated cells to cisplatin.

Experimental

General procedures

$K_2[PtCl_4]$ was purchased from Johnson Matthey and Co. 17α-Ethynylestradiol was obtained from ICN Biomedicals. Deuterated solvents were bought from Euriso-top, France. All other chemicals were obtained from Aldrich and used without further purification. The NMR spectra were recorded at 25 °C on a JEOL Eclipse Plus spectrometer operating at 400 MHz (^{1}H), 100.5 MHz (^{13}C) and 85.9 MHz (^{195}Pt with a spectral window of 1200 ppm). ^{1}H and ^{13}C NMR chemical shifts were reported in parts per million referenced to solvent resonances; for D_2O measurements, 1% methanol was added as a ^{13}C internal reference. ^{195}Pt NMR spectra were recorded using a solution of $K_2[PtCl_4]$ with KCl as an external reference. The shift for $K_2[PtCl_4]$ was adjusted to −1628 ppm from $Na_2[PtCl_6]$ (δ = 0 ppm).

DCI-MS spectra of the ligands were recorded on a Finnigan-MAT 95Q instrument with magnetic and electrostatic analysers. Isobutane was used as the reagent gas at 0.5 mbar pressure. The ion source temperature was kept at 50 °C, the electron emission current at 0.2 mA, and the electron energy at 200 eV. Positive ion spectra were collected. ESI mass spectrometry, due to the soft ionisation, was chosen as an efficient method for the characterisation of platinum complexes in solution.[30] These mass experiments were conducted by means of Finnigan Thermoquest LCQ DUO ion trap mass spectrometer equipped with an ESI ion source. High purity nitrogen was used as a nebuliser (operating pressure at 80 of the arbitrary scale 0–100 of the instrument). The ESI probe tip and capillary potential were set at 4.50 kV and 10.00 V, respectively. The heated capillary was set at 270 °C. The mass spectrometer was operated in positive ion full-scan mode. Molecular ion peaks were assigned from the *m*/*z* values and from the simulated isotope distribution patterns.

Ligand synthesis

Synthesis of *N*-(4-(17α-ethynylestradiolyl)-benzyl)-ethylenediamine, 4.

i) Synthesis of N-(4-iodobenzyl)-ethylenediamine, ***1***. 4-Iodobenzyl bromide (2.86 g, 9 mmol) was dissolved in EtOH (29 ml). A great excess of ethylenediamine 99% (47 mmol, 3.2 ml) was dissolved in the same solvent (10 ml) and added to the first solution while being stirred under a nitrogen atmosphere in the dark. After 24 h the resulting pale yellow mixture was filtered and dried under vacuum, and the oily residue was partitioned between benzene and water. The organic phase was dried over Na_2SO_4 and concentrated to yield *N*-(4-iodobenzyl)-ethylenediamine as a light yellow oil which was used in a subsequent step without further purification. δ_H (400 MHz; $CDCl_3$; Me_4Si) 7.49 (2H, d, $^3J_{b,c}$ 8.2, 2 × Ph*H*b), 6.95 (2H, d, $^3J_{b,c}$ 8.2, 2 × Ph*H*c), 3.59 (2H, s, Ph–CH_2–N), 2.63 (2H, t, ^3J 5.8, N–CH_2–CH$_2$–N), 2.52 (2H, t, ^3J 5.8, N–CH$_2$–CH_2–N), 1.42 (3H, s, N*H* and NH_2); δ_C (100.5 MHz; $CDCl_3$; Me_4Si) 140.25 (Ph*C*a), 137.34 (2 × Ph*C*b), 130.12 (2 × Ph*C*c), 92.23 (Ph*C*d), 53.20 (Ph–*C*H$_2$–N), 51.82 (N–*C*H$_2$–CH$_2$–N), 41.71 (N–CH$_2$–*C*H$_2$–N). DCI-MS: *m*/*z*: 277.0207 ([M + H]$^+$, 100%), 278.0200 (10.70); calcd for $C_9H_{14}IN_2$ 277.0202 ([M + H]$^+$, 100%), 278.0202 (10.68). Elemental analysis calcd for $C_9H_{13}IN_2$: C 39.15; H 4.75; N 10.15; found: C 39.2; H 4.8; N 10.2%.

ii) Synthesis of N-(4-iodobenzyl)-N,N′-diBoc-ethylenediamine, ***2***. A solution of di-*tert*-butyl dicarbonate (Boc_2O, 2.8 g, 12.8 mmol) in CH_2Cl_2 (20 ml) was added to a solution of the above compound in CH_2Cl_2 (3 ml). The reaction mixture was milky at first and then turned a clear yellow. After 24 h stirring at rt, the mixture was dried under vacuum to obtain an oil. Precipitation was induced by *n*-hexane, resulting in an abundant white precipitate **2** (2 g, 42%). δ_H (400 MHz; $(CD_3)_2CO$; Me_4Si) 7.69 (2H, d, $^3J_{b,c}$ 8.2, 2 × Ph*H*b), 7.10 (2H, d, $^3J_{b,c}$ 8.2, 2 × Ph*H*c), 5.84 (1H, s, N*H*), 4.44 (2H, s, Ph–CH_2–N), 3.30 (2H, m, N–CH_2–CH$_2$–N), 3.20 (2H, m, N–CH$_2$–CH_2–N), 1.45 (9H, s, 3 × BocCH_3), 1.40 (9H, s, 3 × BocCH_3); δ_C (100.5 MHz; $(CD_3)_2CO$) 156.07 (Boc*C*=O), 155.76 (Boc*C*=O), 139.43 (Ph*C*a), 137.80 (Ph*C*b), 130.02 (2 × Ph*C*c), 91.97 (Ph*C*d), 79.57 (Boc*C*), 78.15 (Boc*C*), 50.42 (Ph–*C*H$_2$–N), 46.69 (N–*C*H$_2$–CH$_2$–N), 39.27 (N–CH$_2$–*C*H$_2$–N), 28.12 (3 × Boc*C*H$_3$), 28.02 (3 × Boc*C*H$_3$). DCI-MS: *m*/*z* 477.1241 ([M + H]$^+$, 100%), 478.1238 (22.02), 479.1252 (3.15) (M + H)$^+$; calcd for $C_{19}H_{30}IN_2O_4$ 477.1250 ([M + H]$^+$, 100%), 478.1250 (21.89), 479.1250 (3.11). Elemental analysis calcd for $C_{19}H_{29}IN_2O_4$: C 47.9; H 6.1; N 5.9; found: C 48.0; H 6.15; N 5.9%.

iii) Synthesis of N-(4-(17α-ethynylestradiolyl)-benzyl)-N,N′-diBoc-ethylenediamine, ***3***. In a 100 ml three-necked round-bottomed flask, 17α-ethynylestradiol (1.25 g, 4.23 mmol) and **2** (2 g, 4.19 mmol) were dissolved in MeCN (28 ml) and Et_2NH (40 ml). CuI (3% mol/mol respect of the halide and the alkyne, 25 mg, 0.13 mmol) was added to this pale yellow mixture and the mixture turned orange-pink). When $(PPh_3)_2PdCl_2$ (2%, 59 mg, 0.084 mmol) was added, the mixture turned brown-orange. Nitrogen was blown through the solution for 30 min. After being stirred for 5 days at 40 °C, during which time the solution remained a dark brown-orange, the mixture was cooled at rt and dried under vacuum, resulting in an oil that was purified *via* water–CH_2Cl_2 extraction. The organic phase was dried under vacuum to produce a thick dark brown-orange oil which was purified by silica gel column chromatography with *n*-hexane–acetone 2 : 1 to get **3** (2.7 g, 63% from **2**). δ_H (400 MHz; $(CD_3)_2CO$; Me_4Si) 8.27 (1H, s, 3-H), 7.38 (2H, d, ^3J 7.9, 23-H and 27-H), 7.23 (2H, d, ^3J 7.9, 24-H and 26-H), 7.08 (1H, d, $^3J_{1,2}$ 8.4, 1-H), 6.58 (1H, dd, $^3J_{1,2}$ 8.4 and $^4J_{2,4}$ 2.6, 2-H), 6.51 (1H, d, $^4J_{2,4}$ 2.6, 4-H), 6.03 (1H, s, NH), 4.61 (1H, s, 17-H), 4.45 (2H, s, 28-H), 3.21 (4H, m, 29-H and 30-H), 2.74 (2H, m, 6-H and 6′-H), 2.40–2.20 (2H, m, 11-H and 12-H), 2.20–1.90 (3H, m, 16-H, 12′-H and 9-H), 1.90–1.50 (4H, m, 16′-H, 15-H, 14-H, 7-H), 1.50–1.30 (4H, m, 15′-H, 11′-H, 8-H, 7′-H), 1.46 (9H, s, 3 × BocCH_3), 1.38 (9H, s, 3 × BocCH_3), 0.92 (3H, s, 18-H); δ_C (100.5 MHz; $(CD_3)_2CO$; Me_4Si) 156.14 (Boc*C*=O), 155.72 (Boc*C*=O), 155.30 (C-3), 139.20 (C-25), 137.64 (C-5), 131.65 (C-23 and C-27), 131.11 (C-10), 127.91 (C-24 or C-26), 127.47 (C-26 or C-24), 126.32 (C-1), 122.55 (C-22), 115.30 (C-4), 112.96 (C-2), 94.34 (C-20), 84.81 (C-21), 79.91 (Boc*C*), 79.55 (Boc*C*), 78.11 (C-17), 50.87 (C-28), 49.96 (C-14), 47.75 (C-13), 46.38 (C-29 or C-30), 44.03 (C-9), 39.97 (C-8), 39.30 (C-12), 38.94 (C-30 or C-29), 33.31 (C-16), 29.67 (C-6), 27.99 (3 × Boc*C*H$_3$), 27.88 (3 × Boc*C*H$_3$), 27.45 (C-7), 26.67 (C-11), 22.90 (C-15), 12.71 (C-18). DCI-MS: *m*/*z* 645.3895 ([M + H]$^+$, 100%), 646.3911 (43.86), 643.3907 (10.53), 649.3888 (1.85) (M + H)$^+$; calcd for $C_{39}H_{53}N_2O_6$ 645.3903 ([M + H]$^+$, 100%), 646.3903 (43.94), 647.3903 (10.66), 649.3903 (1.86). Elemental analysis calcd for $C_{39}H_{52}N_2O_6$: C 72.6; H 8.1; N 4.3; found: C 72.8; H 8.15; N 4.3%.

iv) Synthesis of N-(4-(17α-ethynylestradiolyl)-benzyl)-ethylenediamine dihydrochloride, ***4***. The above compound (1.5 g, 2.33 mmol) was treated at room temperature with 0.1 N HCl in diethyl ether (12 ml). After 5 days stirring we got a brown precipitate of *N*-(4-(17α-ethynylestradiolyl)-benzyl)-ethylenediamine dihydrochloride (506 mg, 42%). δ_H (400 MHz; D_2O; Me_4Si) 7.22 (2H, d, ^3J 7.3, 23-H and 27-H), 7.17 (2H, d, ^3J 7.3, 24-H and 26-H), 6.72 (1H, d, $^3J_{1,2}$ 5.5, 1-H), 6.40 (1H, d, $^3J_{1,2}$ 5.5, 2-H), 6.17 (1H, s, 4-H), 3.96 (2H, s, 28-H), 3.40–3.07 (4H, m, 29-H and 30-H), 2.22 (2H, m, 6-H), 2.10–0.60 (13H, m, 7-H, 8-H, 9-H, 11-H, 12-H, 14-H, 15-H and 16-H), 0.46 (3H, s, 18-H); δ_C (100.5 MHz; D_2O; Me_4Si) 153.36 (C-3), 137.96 (C-5), 133.62 (C-25), 132.38 (C-23 or C-27), 132.16 (C-27 or C-23), 131.25 (C-10), 130.39 (C-24 or C-26), 130.25 (C-26 or C-24), 126.59 (C-1), 124.11 (C-22), 115.45 (C-4), 112.89 (C-2), 94.09 (C-20), 85.22 (C-21), 80.15 (C-17), 51.40 (C-28), 49.82 (C-14), 47.47 (C-13), 44.07 (C-29 or C-30), 43.39 (C-9), 39.22 (C-8), 35.68 (C-30 or C-29), 33.04 (C-12), 29.23 (C-6), 27.89 (C-16), 27.32 (C-7), 26.22 (C-11), 22.84 (C-15), 12.86 (C-18). DCI-MS:

m/z 223.6505 ([M–2Cl$^-$]$^{2+}$, 100%), 224.1499 (32.63), 224.6507 (5.67) (M–2Cl$^-$)$^{2+}$; calcd for $C_{29}H_{38}N_2O_2$ 223.6503 ([M–2Cl$^-$]$^{2+}$, 100%), 224.1503 (32.75), 224.6503 (5.60). Elemental analysis calcd for $C_{29}H_{38}Cl_2N_2O_2$: C 67.3; H 7.4; N 5.4; found: C 67.4; H 7.4; N 5.4%.

Synthesis of 2-[4-(17α-ethynylestradiolyl)-benzoylamino]-malonic acid diethyl ester, 6.

i) Synthesis of 2-[4-iodobenzoylamino]-malonic acid diethyl ester, 5. To a stirred solution of CDMT (0.88 g, 5 mmol) and 4-iodobenzoic acid (1.26 g, 5 mmol) in CH_2Cl_2 (7 mL), we added *N*-methylmorpholine (0.52 g, 5 mmol) dropwise at −5 °C. After 2 h a TLC control indicated the consumption of CDMT. To the crude solution obtained as described above, a mixture of diethyl 2-aminomalonate hydrochloride (1.06 g, 5 mmol) and *N*-methylmorpholine (0.52 g, 5 mmol) in CH_2Cl_2 (7 mL) was added at −5 °C. After 2 h at 0 °C the temperature was brought to rt and the reaction stirred for 14 h. Evaporation of the solvent resulted in a residue that was suspended in ethyl acetate (30 mL). The organic layer was successively washed with H_2O (10 mL), a 10% citric acid solution (10 mL), H_2O (10 mL), saturated $NaHCO_3$ solution (10 mL) and H_2O (10 mL). Anhydrification of the organic phase with $MgSO_4$ and evaporation of the solvent produced a residue that was recrystallised from ethyl acetate, resulting in 1.45 g (71.5% yield) of pure **5**. δ_H (400 MHz; $(CD_3)_2CO$; Me_4Si) 8.24 (1H, d, ^3J 7.2, N*H*), 7.89 (2H, d, $^3J_{b,c}$ 8.6, 2 × Ph*H*c), 7.74 (2H, d, $^3J_{b,c}$ 8.6, 2 × Ph*H*b), 5.36 (1H, d, ^3J 7.2, N–C*H*), 4.23 (4H, m, 2 × OCH_2CH$_3$), 1.26 (6H, t, ^3J 7.1, 2 × OCH$_2$CH_3); δ_C (100.5 MHz; $(CD_3)_2CO$; Me_4Si) 166.55 (2 × C-*C*=O), 166.06 (N–*C*=O), 137.95 (2 × Ph*C*c), 133.35 (Ph*C*a), 129.62 (2 × Ph*C*b), 98.63 (Ph*C*d), 62.13 (2 × O*C*H$_2$CH$_3$), 57.08 (N–*C*H), 13.67 (2 × OCH$_2$*C*H$_3$). DCI-MS: *m/z* 406.0149 ([M + H]$^+$, 100%), 407.0146 (15.84), 408.0156 (2.18); calcd for $C_{14}H_{17}INO_5$ 406.0151 ([M + H]$^+$, 100%), 407.0151 (15.96), 408.0151 (2.22). Elemental analysis calcd for $C_{14}H_{16}INO_5$: C 41.5; H 4.0; N 3.5; found: C 41.6; H 4.0; N 3.5%.

ii) Synthesis of 2-[4-(17α-ethynylestradiolyl)-benzoylamino]-malonic acid diethyl ester, 6. In a 50 ml three-necked round-bottomed flask, anhydrous THF (10 mL) was degassed with N_2 for 10 min. Then, Ag_2O (0.35 g, 1.5 mmol), 17α-ethynylestradiol (0.44 g, 1.5 mmol), **5** (0.61 g, 1.5 mmol) and $(PPh_3)_2PdCl_2$ (52.6 mg, 0.075 mmol) were subsequently added. The reaction mixture was heated to 60 °C and stirred under nitrogen overnight. The reaction was then cooled to rt, filtered through a filter agent, and the solid washed with diethyl ether. The organic phase was washed with $NaHCO_3$ 5% (10 mL) and brine (10 mL), dried over $MgSO_4$ and the solvent was evaporated. The residue was purified by column chromatography using petroleum ether–diethyl ether 50 : 50 as eluant. Appropriate fractions were combined giving 0.53 g (61.1% yield) of pure **6**. δ_H (400 MHz; $(CD_3)_2CO$; Me_4Si) 8.24 (1H, d, ^3J 7.3, N*H*), 7.99 (1H, s, 3-H), 7.94 (2H, d, ^3J 8.4, 24-H and 26-H), 7.54 (2H, d, ^3J 8.4, 23-H and 27-H), 7.10 (1H, d, $^3J_{1,2}$ 8.4, 1-H), 6.60 (1H, dd, $^3J_{1,2}$ 8.4 and $^4J_{2,4}$ 2.6, 2-H), 6.53 (1H, d, $^4J_{2,4}$ 2.6, 4-H), 5.38 (1H, d, ^3J 7.3, 29-H), 4.61 (1H, s, 17-H), 4.24 (4H, m, 2 × OCH_2CH$_3$), 2.75 (2H, m, 6-H and 6′-H), 2.38–2.31 (2H, m, 11-H and 12-H), 2.24–1.92 (3H, m, 16-H, 12′-H and 9-H), 1.92–1.72 (4H, m, 16′-H, 15-H, 14-H, 7-H), 1.52–1.30 (4H, m, 15′-H, 11′-H, 8-H, 7′-H), 1.26 (6H, t, ^3J 7.1, 2 × OCH$_2$CH_3), 0.94 (3H, s, 18-H); δ_C (100.5 MHz; $(CD_3)_2CO$; Me_4Si) 166.46 (*C*=O, C-30 and C-31), 165.98 (*C*=O, C-28), 155.15 (C-3), 137.65 (C-5), 132.67 (C-25), 131.48 (C-23 and C-27), 131.12 (C-10), 127.78 (C-24 and C-26), 127.24 (C-22), 126.34 (C-1), 115.21 (C-4), 112.87 (C-2), 97.09 (C-20), 84.19 (C-21), 79.54 (C-17), 62.03 (2 × O*C*H$_2$CH$_3$), 56.95 (C-29), 50.00 (C-14), 47.76 (C-13), 43.90 (C-9), 39.86 (C-8), 39.21 (C-12), 33.32 (C-16), 29.58 (C-6), 27.43 (C-7), 26.64 (C-11), 22.87 (C-15), 13.57 (2 × OCH$_2$*C*H$_3$), 12.67 (C-18). DCI-MS: (*m/z* (relative intensity)): 574.2808 ([M + H]$^+$, 100%), 575.2797 (38.09), 576.2796 (8.47), 577.2812 (1.38); calcd for $C_{34}H_{40}NO_7$ 574.2804 ([M + H]$^+$, 100%), 575.2804 (38.01), 576.2804 (8.46), 577.2804 (1.39). Elemental analysis calcd for $C_{34}H_{39}NO_7$: C 71.2; H 6.85; N 2.4; found: C 71.3; H 6.9; N 2.4%.

Platinum coordination

Synthesis of *cis*-dichloro[*N*-(4-(17α-ethynylestradiolyl)-benzyl)-ethylenediamine]platinum(II), 7. The ligand–dihydrochloride **4** (132 mg, 0.255 mmol) was dissolved in water (5 ml) at 65 °C. A solution of $K_2[PtCl_4]$ (127 mg, 0.306 mmol) in water (5 ml) was added and the pH value was adjusted to 5–6 with 0.1 N aqueous NaOH. Initially, the pH of the mixture was 4–5 decreasing towards 1 because of the progressing reaction; the pH had to be kept from the region of hydroxocomplexes (basic pH). The mixture was stirred while protected from light for 2 days at rt. The resulting light brown precipitate of **7** was washed with cold water, ethanol and ether and dried, resulting in pure **7** (160 mg, 88% from **4**). δ_H (400 MHz; $(CD_3)_2SO$; Me_4Si) 8.99 (1H, s, 3-H), 7.58 (2H, d, ^3J 7.9, 24-H and 26-H), 7.45 (2H, d, ^3J 7.9, 23-H and 27-H), 7.06 (1H, d, $^3J_{1,2}$ 8.4, 1-H), 6.70 (1H, brs, N*H*), 6.51 (1H, d, $^3J_{1,2}$ 8.4, 2-H), 6.44 (1H, s, 4-H), 5.45 (1H, s, 17-H), 5.43–5.23 (2H, m, NH_2), 4.60–3.60 (6H, m, 28-H, 29-H and 30-H), 2.71 (2H, m, 6-H), 2.45–1.13 (13H, m, 7-H, 8-H, 9-H, 11-H, 12-H, 14-H, 15-H and 16-H), 0.82 (3H, s, 18-H); δ_C (100.5 MHz; $(CD_3)_2SO$; Me_4Si) 155.01 (C-3), 137.91 (C-5), 134.32 (C-10), 131.49 (C-23 or C-27), 130.52 (C-27 or C-23), 130.35 (C-24 or C-26), 130.26 (C-26 or C-24), 128.39 (C-25), 126.18 (C-1), 123.09 (C-22), 115.00 (C-4), 112.82 (C-2), 95.90 (C-20), 83.74 (C-21), 78.72 (C-17), 53.95, 53.16, 45.32 (N–CH$_2$, C-28, C-29, C-30), 49.50 (C-14), 47.33 (C-13), 43.49 (C-9), 33.02 (C-16), 29.27 (C-6), 27.08 (C-7), 26.30 (C-11), 22.66 (C-15), 12.97 (C-18); δ_{Pt} (85.9 MHz; $(CD_3)_2SO$; $K_2[PtCl_4]$) −3307 ppm (where a chloride is replaced by DMSO). ESI-MS (water–DMSO (3 : 1)): *m/z* 749.221 (1.52%), 751.221 (64.40), 752.220 (88.58), 753.220 ([M–Cl + DMSO]$^+$, 100), 754.220 (54.73), 755.221 (46.76), 756.221 (14.95), 757.220 (8.66), 758.221 (2.44); calcd for $C_{31}H_{42}ClN_2O_3PtS$ 749.220 (1.53), 751.220 (64.46%), 752.220 (88.53), 753.220 ([M–Cl + DMSO]$^+$, 100), 754.220 (54.77), 755.220 (46.74), 756.220 (14.97), 757.220 (8.63), 758,220 (2.45). Elemental analysis calcd for $C_{29}H_{36}Cl_2N_2O_2Pt$: C 49.0; H 5.1; N 3.9; Pt 27.45; found: C 49.1; H 5.1; N 3.9; Pt 27.4%.

Synthesis of *cis*-diamino[2-(4-(17α-ethynylestradiolyl)-benzoylamino)-malonato]platinum(II), 8. $K_2[PtCl_4]$ (500 mg, 1.21 mmol) was dissolved in water (2 ml) and an aqueous solution of KI (1.20 mg, 7.23 mmol of KI in 2 ml of water) was added in the dark to the mixture. After about 30 min it was filtered to remove some solid impurities. An aqueous solution (2 ml) containing 4 mmol of NH_3 was added to the filtrate. Fine yellow-brown crystals of *cis*-$[(NH_3)_2PtI_2]$ immediately precipitated. After 15 min the compound was separated by centrifugation and washed with cold water, ethanol and diethyl ether (567 mg, 97.5% yield). *cis*-$[(NH_3)_2PtI_2]$ (300 mg, 0.62 mmol) was then suspended in an aqueous solution (30 ml) of Ag_2SO_4 (190 mg, 0.61 mmol) and the mixture was stirred for 20 h in the dark; after that the silver iodide precipitate was removed by filtration. The barium salt **6a** [prepared by adding 0.11 M aqueous $Ba(OH)_2$ (5.65 ml) to a solution of **6** (400 mg, 0.70 mmol) in methanol (10 ml)] was added to the filtrate. The mixture was stirred for 24 h and then centrifugated to separate the $BaSO_4$ and **8** that coprecipitated. The solid obtained was washed several times with methanol to dissolve **8**. Methanol was evaporated to dryness, yielding **8** (250 mg, 54% yield from *cis*-$[(NH_3)_2PtI_2]$). δ_H (400 MHz; $(CD_3)_2SO$; Me_4Si) 9.01 (1H, s, 3-H), 7.89 (1H, d, ^3J 7.7, N*H*), 7.86 (2H, d, ^3J 8.4, 24-H and 26-H), 7.49 (2H, d, ^3J 8.4, 23-H and 27-H), 7.06 (1H, d, $^3J_{1,2}$ 8.4, 1-H), 6.49 (1H, dd, $^3J_{1,2}$ 8.4 and $^4J_{2,4}$ 2.6, 2-H), 6.42 (1H, d, $^4J_{2,4}$ 2.6, 4-H), 5.72 (1H, d, ^3J 7.7, 29-H), 5.55 (1H, s, 17-H), 4.31 (6H, s, 2 × NH_3), 2.70 (2H, m, 6-H and 6′-H), 2.40–1.20 (13H, m, 7-H, 8-H, 9-H, 11-H, 12-H, 14-H, 15-H and 16-H), 0.80 (3H, s, 18-H); δ_C (100.5 MHz; $(CD_3)_2SO$; Me_4Si) 171.73, 170.48 (*C*=O, C-30 and C-31), 164.39 (*C*=O,

C-28), 155.00 (C-3), 137.28 (C-5), 133.51 (C-10), 131.31 (C-23 and C-27), 130.39 (C-25), 127.78 (C-22), 127.64 (C-24 and C-26), 126.24 (C-1), 115.03 (C-4), 112.86 (C-2), 97.17 (C-20), 83.78 (C-21), 78.80 (C-17), 59.47 (C-29), 49.56 (C-14), 47.38 (C-13), 43.47 (C-9), 33.08 (C-16), 29.32 (C-6), 27.09 (C-7), 26.34 (C-11), 22.72 (C-15), 13.01 (C-18); δ_{Pt} (85.9 MHz; $(CD_3)_2SO$; $K_2[PtCl_4]$) −1718 ppm. ESI-MS (water–methanol = 50 : 50): *m/z* 742.214 (1.78%), 744.215 (73.08), 745.215 ($[M + H]^+$, 100), 746.214 (86.88), 747.215 (25.31), 748.216 (20.76), 749.215 (6.19), 750.216 (1.22); calcd for $C_{30}H_{36}N_3O_7Pt$ 742.215 (1.73%), 744.215 (73.11), 745.215 ($[M + H]^+$,100), 746.215 (86.85), 747.215 (25.38), 748.215 (20.80), 749.215 (6.18), 750.215 (1.23). Elemental analysis calcd for $C_{30}H_{35}N_3O_7Pt$: C 48.4; H 4.7; N 5.6; Pt 26.2; found: C 48.5; H 4.7; N 5.6; Pt 26.15%.

Log $P_{o/w}$ determination

Log $P_{o/w}$ values were estimated from values of log k'_w (k'_w = capacity ratio in absence of methanol), determined by HPLC chromatography according to Minick *et al.*[54] and Pomper *et al.*[55] 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-morpholinopropanesulfonic acid (MOPS) and 0.15% (v/v) *n*-decylamine; the pH was adjusted to 7.4 with NaOH. The flow rate was 0.5 ml min^{-1}. The steroids were dissolved at 1 mM concentration in ethanol and 20 µl were injected. Column void volume was estimated from the retention time of uracil (t_0), which was included as an unretained internal reference with each run. k' values 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' *vs.* ϕ methanol (ϕ = volume fraction of methanol); data acquired with $0.55 \leq \phi \leq 0.85$. Applying Pomper's relationship, log k' returned the log $P_{o/w}$ value.

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

Lamb 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 the literature[56] was used as a source of ERα.

Aliquots (200 µL) of diluted ERα (in glass tubes) were incubated for 3.5 h at 0 °C with 2×10^{-9}M of [6,7-^{3}H]-estradiol (specific activity 1.96 TBq $mmol^{-1}$) in the presence of nine different concentrations of the hormone derivative to be tested. At the end of the incubation period, the free and bound fractions of the tracer were separated by protamine sulfate precipitation.[42] The percentage reduction of [^{3}H]-estradiol binding (Y) was calculated using the logit transformation of Y (logit $Y = \ln[Y/1 - Y]$) *versus* the logarithm of the mass of the competing steroid. The unlabelled steroid concentration, requiring displacement of 50% of the bound [^{3}H]-estradiol, was calculated for each steroid tested, and the results are expressed as RBA. The RBA value of estradiol is by definition equal to 100%.

Viability assay

Exponentially growing cell lines, the MCF-7 hormone-responsive mammary adenocarcinoma cell line and MDA-MB-231 hormone-independent breast cancer line (ATTC, USA), were trypsinized and resuspended in complete RPMI (Sigma, USA) culture medium phenol red-free, containing 10% charcoal–dextran stripped FBS (2.5 g of washed, activated charcoal, plus 0.25 g dextran T-70 dissolved in 1 L of 0.01 M Tris, pH 8.0, added to an equal volume of fetal bovine serum) (Sigma, USA), supplemented with 2 mM L-glutamine, 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin and 0.25 µg ml^{-1} amphotericin B. Cells were seeded with complete media conditioned with an appropriate concentration of the compound under analysis (final concentration 1 µM). The organic compounds Tam, OH–Tam, E2, EE, **4** and **6**, were dissolved in EtOH, whereas Pt(II)-complexes **7**, **8**, *cis*-[Pt(en)Cl_2] and *cis*-[Pt(NH_3)mal] were dissolved in DMSO. The final concentration of the cosolvent in culture media never exceeded 0.5%. Cisplatin was dissolved in culture media. Cell monolayers grown with culture media containing the cosolvent at the maximum concentration employed were assumed as reference controls (100% of the growth).

Cell monolayers were treated continuously for 120 h. Viability was measured using an MTT test. Cells were incubated with 0.5 mg ml^{-1} MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) under standard culture conditions for 1 h. Wells were dried, and formazan crystals were soluted with 750 µL of acidic isopropanol (0.1 N HCl in absolute isopropanol). Absorbance was measured by spectrophotometry at 570 nm with background subtraction at 630 and 690 nm.

Bio-stoichiometry

Rough calculations allowed us to assess the order of magnitude of platinum atoms potentially bound to the receptor or necessary to exert cytotoxic effects. The number of estrogen-receptor (ER) adducts in hormone dependent (ER+) breast cancer cells is in the range of 10 000–30 000 (20 000 on average).[57] The level of cellular uptake in the human mammary adenocarcinoma MCF-7 cell line with cisplatin, evaluated by means of ICP-MS and extrapolated at IC_{50} level (24 h continuous treatment), is about 47 ng Pt/10^6 cells.[58] The corresponding DNA platination is about 1% of the uptake, *i.e.* 470 pg Pt/10^6 cells.[59,60] Provided that the 20 000 ER adducts transport 20 000 Pt atoms directly to the DNA (vehiculation perfectly efficient), the DNA platination would correspond to 6.4 pg Pt/10^6 cells. Thus, the platination produced by the receptorial system proves to be sorely inadequate for cytotoxicity, with a deficit of at least 73-fold.

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