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Inhibition of human topoisomerase I and II and anti-proliferative effects on MCF-7 cells by new titanocene complexes

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ABSTRACT

The antitumor activity shown by many platinum complexes has produced a strong interest in research of new organometallic compounds having anticancer action. Among the many metal compounds synthesized and tested, those based on titanium have received considerable attention because of their cytotoxic activity against solid tumors. Particularly, new titanocene compounds containing aromatic groups linked to the Cp (cyclopentadienyl ring, C_5H_5) have been synthetized, such as the titanocene Y (bis-[(*p*-methoxybenzyl)cyclopentadienyl]titanium dichloride) that displayed promising medium–high cytotoxic activity on breast cancer cell lines. Other titanocene complexes recently synthesized, obtained by replacing the substituent methoxy-aryl of cyclopentadienes of titanocene Y with ethenyl-methoxide or ethenyl-phenoxide, showed increased cytotoxic activity on breast cancer cell lines being more stable compounds. In this paper, we report that new titanocene complexes holding lipophilic groups, for instance a methyl group on benzyl carbon, exhibit improved antiproliferative effect on breast cancer cell line MCF-7. Similar results have been obtained introducing a 5-methoxy naphthyl group to further stabilize the titanocene complexes. These inhibitory effects on breast cancer cells have been ascribed to human topoisomerase I and II inhibition as demonstrated by specific enzymatic assays.

1. Introduction

Several platinum agents, such as *cis*-platin, which exert antiproliferative activity in breast cancer targeting DNA,[1] have produced a strong interest in research of new organometallic compounds as pharmacological anticancer tools. Even though *cis*-platin and platinum-based drugs exhibit high antitumor activities against a wide range of human tumors, the onset of toxic side effects and/or chemoresistance represents the principal limitation to their therapeutic efficacy.[2,3] It has been already reported that DNA is a major target for *cis*-platin even though only a small fraction (5–10%) of intracellular *cis*-platin binds DNA, whereas the major amount (75–85%) has been found bound to other intracellular targets, such as enzymes and RNA.[4,5] This aspecific binding to non-DNA targets could represent a valid explanation for the onset of resistance and, as well, for its high toxicity. Moreover, several studies revealed that *cis*-platin induces the formation of oxygen reactive species (ROS), responsible for its toxic side effects, for example, nephrotoxicity, hepatotoxicity and neurotoxicity.[6]

The antitumor properties of different metal complexes have been evaluated and, amongst them, titanium complexes have received considerable attention because of their cytotoxic activity against solid tumors.[7] It has been proposed that such complexes may interact with DNA and inhibit cell cycle, although the antitumor mechanism depends on the transport and delivery of

Abbreviations: DMSO, dimethyl sulfoxide; THF, tetrahydrofuran; ADP, adenosine diphosphate; ATP, adenosine triphosphate; DAPI, 4′,6-diamidino-2-phenylindole; DMEM/F-12, Dulbecco's modified eagle medium: nutrient mixture F-12; DNA, deoxyribonucleic acid; EDTA, ethylenediaminetetraacetic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAD, nicotinamide adenine dinucleotide; Parp-1, poly(ADP-ribose)polymerase 1; RNA, ribonucleic acid.

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Ti species into cancer cells. Furthermore, the hydrolysis rate plays a major role for the tumor-inhibiting potency, because it is correlated to the interaction with nucleic acids, proteins and other potential intracellular targets.[8] As a matter of fact, budotitane [*cis*-diethoxybis (1-phenylbutane-1,3-dionate)titanium(IV)] and the titanocene dichloride (Cp_2TiCl_2, TDC) (Fig. 1), showed very promising results in preclinical studies, but did not pass the phase I and II clinical trials.[9] It has been observed a high rate of hydrolysis at neutral pH and polymeric hydrolysis products precipitation.[10,11] Additionally, these compounds showed a modest efficacy in patients with metastatic breast cancer.[12,13]

Literature data reported that the titanocene dichloride, differently from *cis*-platin, interacts with phosphate groups and with *N*-sites of bases forming strong DNA adducts, which have been detected in tumor cells, at pH values lower than 5 and weaker at physiological pH values. Furthermore, titanium(IV) binds strongly to protein transferrin, displacing iron(III), may mediate the uptake of Ti into cancer cells.[10]

Thus, these outcomes encouraged the development of new titanocene complexes having a higher hydrolytic stability and higher cytotoxic activity introducing polar side chains attached to the Cp ligands, for example, alkoxo, amino or carboxylic acid and esters,[14–20] and also other titanocene derivatives containing aromatic groups linked to the Cp have been prepared.[20]

One of the most interesting of these compounds, namely the titanocene Y (bis-[(*p*-methoxybenzyl)-cyclopentadienyl]-titanium-dichloride), (Fig. 1), showed antiproliferative activity in numerous human tumor cell lines[14,21] and in explanted human tumors, particularly in MCF-7 with a medium–high cytotoxic activity (IC_{50} of 76 μM).[16,22–24]

Titanocene complexes obtained by replacing the substituent methoxy-aryl of cyclopentadienes of titanocene Y with ethenyl-methoxide or ethenyl-phenoxide were recently synthesized and tested.[17,19]

Particularly, the complexes bis-[methoxy-ethenyl-cyclopentadienyl]-titanium-dichloride (**T_2**), bis-[phenoxy-ethenyl-cyclopentadienyl]-titanium-dichloride (**T_4**) and [methoxy-ethenyl-cyclopentadienyl]-titanium trichloride (**T_1**) (Fig. 1) showed a good cytotoxicity, very similar or better to *cis*-platin on MCF-7, comparable to the ones reported for titanocene Y.[14]

It has been also verified the influence of leaving ligands on the activity by substituting chlorine atoms with dimethylamide, oxalate or aminoacid groups. The results of the hydrolysis of our titanocenes showed unequivocally that the leaving groups (Cl, $N(CH_3)_2$, C_2O_4 or glycine) significantly affect even the hydrolysis rate of cyclopentadienyl groups, being chloride and oxalate more stable.[17]

Complex **T_1** was the first example of half-titanocene complex that showed interesting cytotoxic activity, and afterwards, other analog complexes synthesized and tested as antiproliferative agents on MCF-7 and SkBr-3 breast cancer cells.[17] These data highlighted the importance of coordinating substituents on cyclopentadienyl ligands for the cytotoxic activity.[19]

Although generalizations regarding structure–activity relationships are not yet clear, it could be assumed that the neutral nucleophilic substituents of cyclopentadienyl (aryl methoxy or

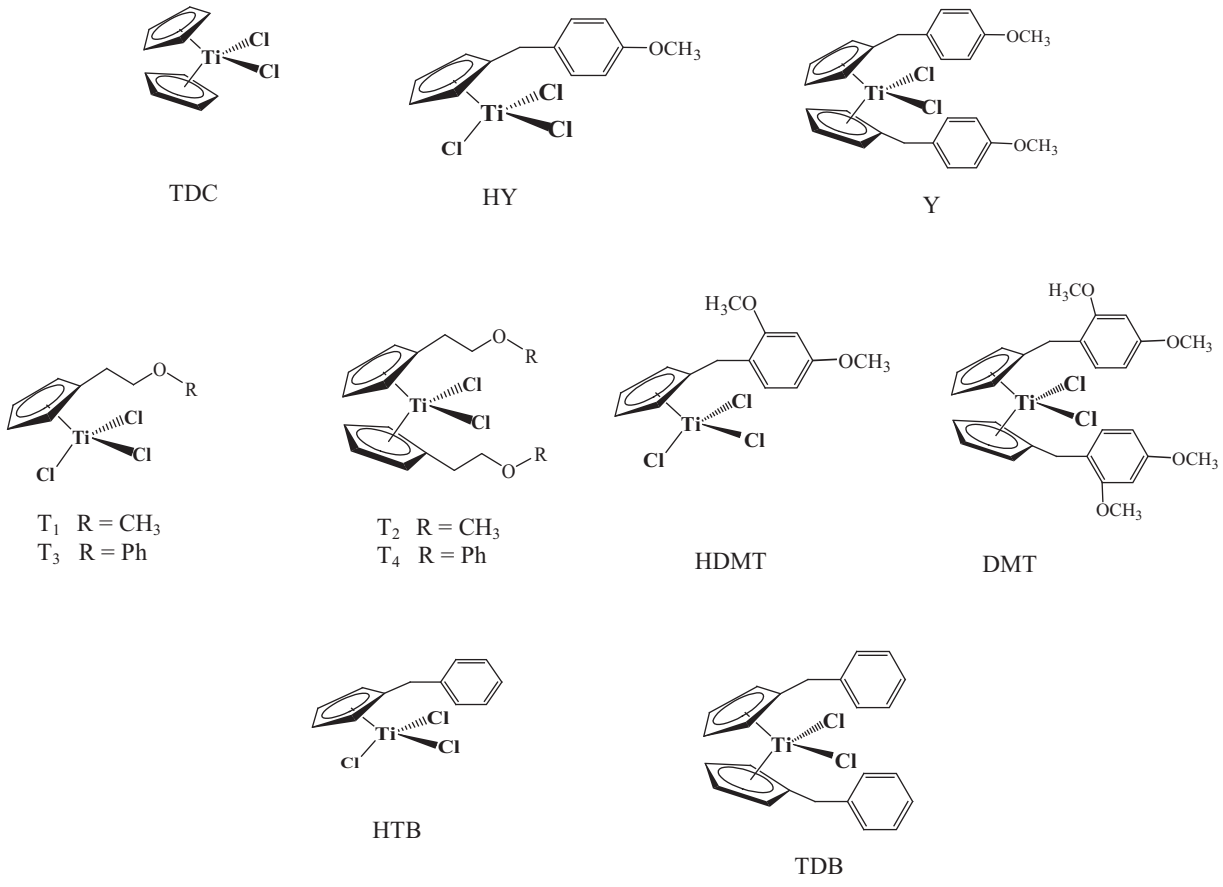


Figure 1. (a) Titanocene dichloride (**TDC**); [(*p*-methoxybenzyl)cyclopentadienyl]titanium trichloride (**HY**), Bis-[(*p*-methoxybenzyl)cyclopentadienyl]titanium dichloride (**Y**); [methoxy-ethenyl-cyclopentadienyl]-titanium-trichloride (**T_1**) bis-[methoxy-ethenyl-cyclopentadienyl]-titanium-dichloride (**T_2**), [phenoxy-ethenyl-cyclopentadienyl]-titanium trichloride (**T_3**), bis-[phenoxy-ethenyl-cyclopentadienyl]-titanium-dichloride (**T_4**), [(2,4-dimethoxybenzyl)cyclopentadienyl]titanium trichloride (**HDMT**), Bis-[(2,4-dimethoxybenzyl)cyclopentadienyl]titanium dichloride (**DMT**); [cyclopentadienyl-benzyl]titanium trichloride (**HTB**), bis-[cyclopentadienyl-benzyl]titanium dichloride (**TB**).

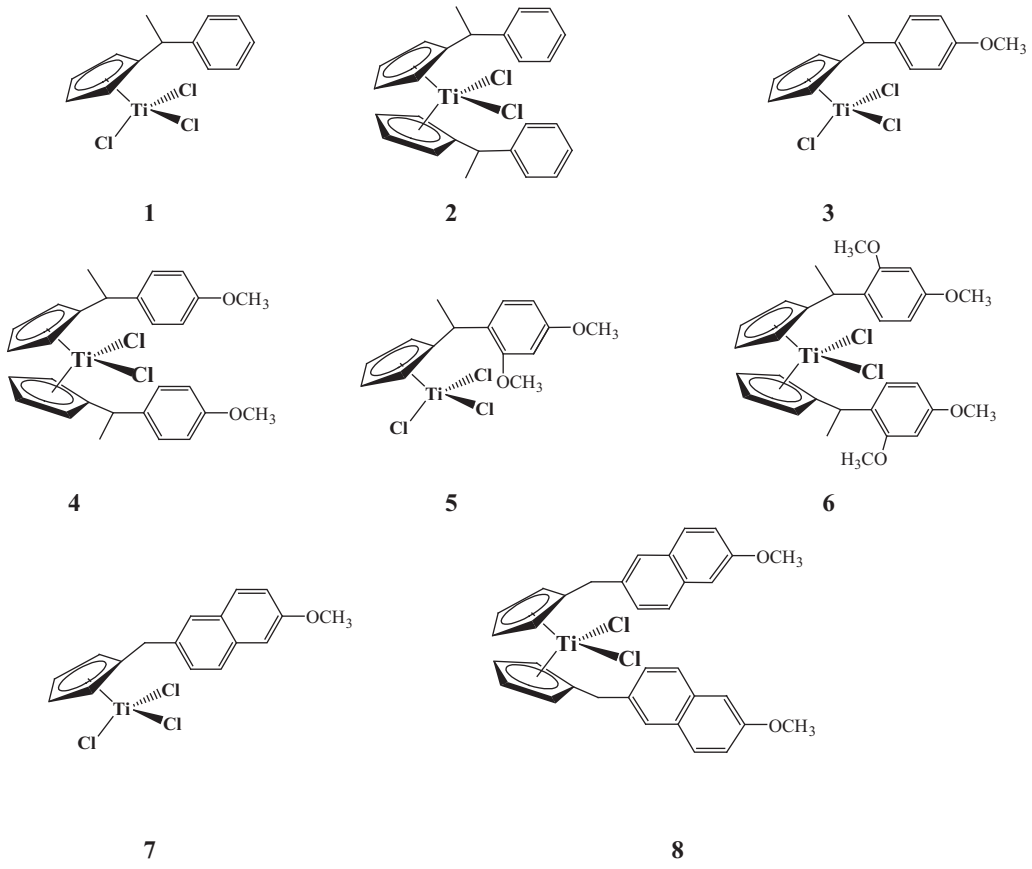


Figure 2. [(Cyclopentadienyl-ethyl-1-benzene)]-titanium-trichloride [$CpCH(CH_3)Ph]TiCl_3$ (**1**), bis[(cyclopentadienyl-ethyl-1-benzene)]-titanium-dichloride [$CpCH(CH_3)Ph]_2TiCl_2$ (**2**), [(cyclopentadienyl-ethyl-1-(4-methoxy-benzene)]-titanium-trichloride [$CpCH(CH_3)(p\text{-}C_6H_4\text{—}OCH_3)]TiCl_3$ (**3**), bis-[(cyclopentadienyl-ethyl-1-(4-methoxy-benzene)]-titanium-dichloride [$CpCH(CH_3)(p\text{-}C_6H_4\text{—}OCH_3)]_2TiCl_2$ (**4**), [(cyclopentadienyl-ethyl-1-(2,4-dimethoxy-benzene)]-titanium-trichloride [$CpCH(CH_3)(C_6H_3(OCH_3)_2)]TiCl_3$ (**5**), bis-[(cyclopentadienyl-ethyl-1-(2,4-dimethoxy-benzene)]-titanium-dichloride [$CpCH(CH_3)(C_6H_3(OCH_3)_2)]_2TiCl_2$ (**6**), [(cyclopentadienyl)-methylene-(5-methoxy-naphtalene)]-titanium-trichloride [$CpCH_2(C_{10}H_6\text{—}OCH_3)]TiCl_3$ (**7**), and bis-[(cyclopentadienyl)-methylene-(5-methoxy-naphtalene)]-titanium-dichloride [$CpCH_2(C_{10}H_6\text{—}\ OCH_3)]_2TiCl_2$ (**8**).

ethenyl-methoxy groups) could intramolecularly coordinate to the titanium cation, thus preventing decomposition reactions. On the other hand, this hypothesis was suggested for analogous complexes able to give polymerization of propene or styrene having microstructures strongly influenced by the possible coordination of neutral substituent of cyclopentadienyl at the metal center.[25–27]

In this work, we report the synthesis, the characterization and the evaluation of the hydrolytic stability of some new titanocene and half-titanocene compounds having a methyl group on the carbon 6 and a methoxy-naphthyl group as substituent of the cyclopentadienyl (Fig. 2). We also tested the antiproliferative effects exerted by these new complexes on human breast cancer cell line MCF-7, evidencing that they act as inhibitors of important DNA-metabolizing enzymes, that is, topoisomerase I and II. The outcomes suggest that these complexes could represent a valid alternative to the most used *cis*-platin, being itself a topoisomerases inhibitor with a proven clinical efficacy, but affecting, as well, the proliferation of normal breast cells.

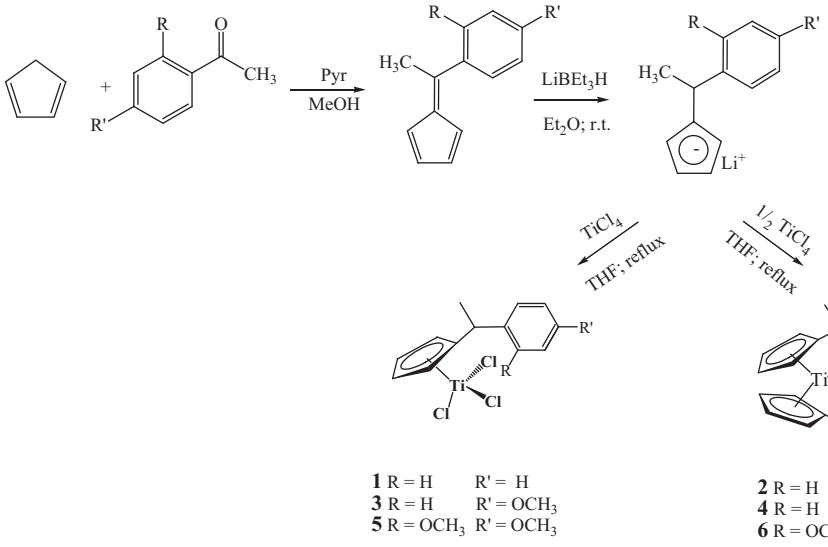
2. Results and discussion

2.1. Chemistry

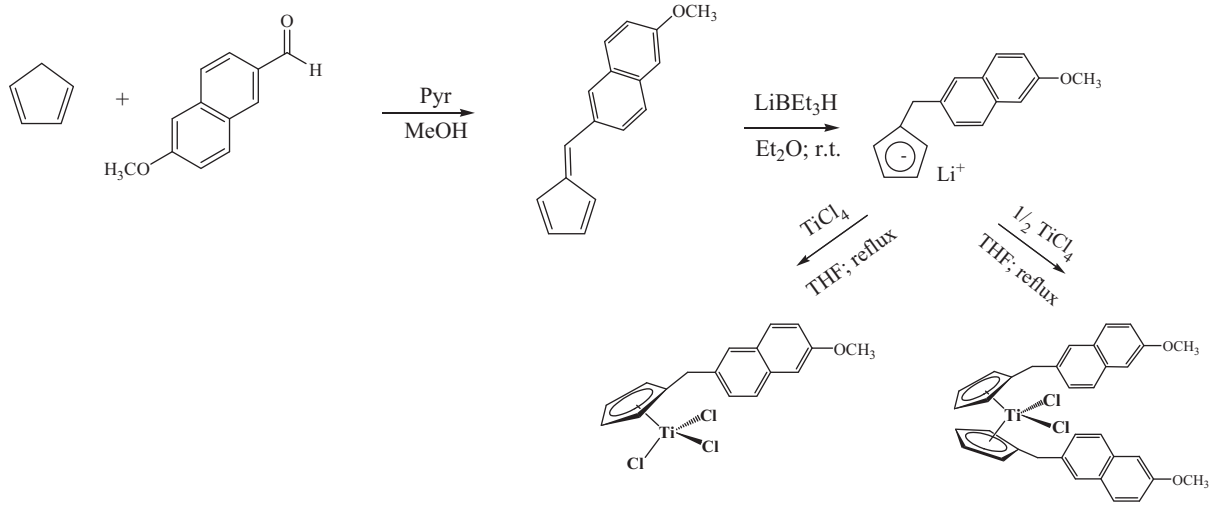
The new titanocene complexes herein reported (**1**–**8**) have been obtained through different methodological synthetic approaches (Fig. 2). Complexes **1**–**6** were synthesized with an additional methyl group on benzyl carbon, in order to increase their lipophilicity with respect to the already reported complexes with cyclopentadienyl-benzyl ligands. Complexes **7** and **8** were synthesized because the group 5-methoxy naphthyl could further stabilize the titanocene complexes and give greater coordinating ability to the reactive sites of DNA than the compounds HY, Y, HDMT and DMT, moreover they are more lipophilic than the latter. In general, the synthesis of **1**–**6** was carried out with good yields by reaction of the lithium salt of the suitable ligand, obtained by reaction of acetophenone derivatives with cyclopentadiene, with the stoichiometric amount of $TiCl_4{\cdot}2THF$ (see Scheme 1).[28]

Complexes **7** and **8** were prepared according to the Scheme 2. The synthesis of the 5-methoxy-naphthyl-fulvene was carried out as already reported in the literature[28] and its lithium salt was obtained by reaction with Super Hydride ($LiBEt_3H$) in dry diethyl ether. Then, it was isolated and subsequently reacted with the suitable amount of $TiCl_4{\cdot}2THF$ in dry THF.

Hydrolytic stability of the titanocene complexes has been determined in aqueous solution, 90% DMSO by ^1H NMR spectroscopy, in order to correlate the chemical stability of these complexes with their potential cytotoxic activity. Since we can expect that rapid hydrolysis of the leaving group (Cl) and cyclopentadienyl ligands could give way to biologically inactive species, whereas active species could be generated if the Cp rings remain metal bound. The hydrolysis of aromatic rings was evaluated by the integration of two signals of protons of cyclopentadienyl bound to metal, compared to newly formed multiplet of substituted cyclopentadiene. The obtained results (Table 1) showed that, among all the



Scheme 1. Synthetic route for the preparation of complexes **1**–**6**.



Scheme 2. Synthetic route for the preparation of complexes **7** and **8**.

Table 1
Hydrolysis results of complexes in DMSO/D_2O solution at room temperature followed by ^1H NMR

Compound	% Cp rings hydrolysis			
	5 min	4 h	8 h	24 h
1	15	30	55	55
2	14	31	52	55
3	<1	<1	<1	<5
4	<1	<1	<1	<5
5	<1	<1	<1	<5
6	<1	<1	<1	<5
7	<1	<1	<1	<5
8	<1	<1	<1	<5

synthesized titanocene complexes, only two (compounds **1** and **2**) resulted more rapidly hydrolysable, probably because of the absence of further coordinating groups (methoxy groups) on the phenyl or naphthyl rings.

2.2. Biological activity

The effects of all new synthesized titanocene complexes on tumor cells viability have been evaluated against estrogen receptor positive (ER+) human breast cancer cell line MCF-7 (Fig. 3A and B) at different concentrations (1, 5, 10, 20, 40 μM) using the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) MTT assay.[29–32] MCF-7 cells were treated for 72 h with each compounds using *cis*-platin as anticancer reference molecule. As reported in Figure 3A, among the tested compounds **1**, **3**, **5** and **7**, only **7** displays antiproliferative activity in a dose-dependent manner. Notably, as shown in Figure 3B, the complex **4** is the most active, with an antiproliferative activity comparable to that of *cis*-platin (Table 2). Compound **2** (Fig. 3B) determined a clear dose–response inhibitory effect on cell growth, while **8** (Fig. 3B) also elicited an inhibition, but only at higher concentrations (40 μM). Compound **6** resulted in a slight but statistically significant reduction of cell vitality, but only at 20 and 40 μM. These considerations indicate that the higher rate of hydrolysis of compound **2** clearly influence

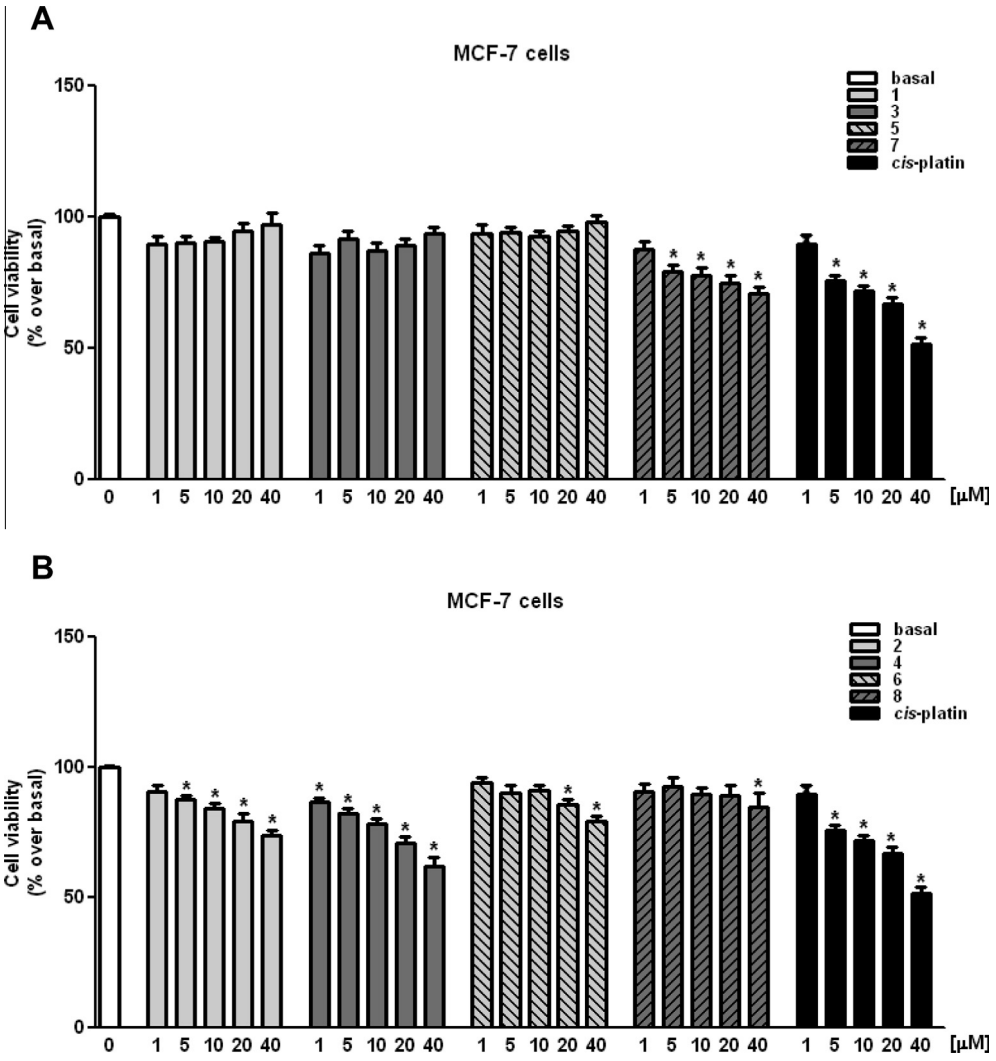


Figure 3. Effects of different doses of titanocene complexes on MCF-7 cell proliferation. Cells were treated for 72 h with the indicated concentrations of half-titanocenes (A) or titanocenes (B). Cell viability was evaluated by MTT assay. Graphs represent mean + SE of three independent experiments each performed in triplicate. Statistically significant differences are indicated (*, P <0.05 vs basal).

Table 2
IC_{50} of *cis*-platin and titanocene complexes for MCF-7 cells

Compounds	IC_{50} (μM)	95% confidence intervals
1	505.6	372.1–686.8
2	85.26	80.13–90.71
3	273.7	221.8–337.8
4	49.16	46.00–52.54
5	664.6	499.6–884.0
6	129.8	122.0–138.0
7	63.13	57.70–69.08
8	171.8	153.8–191.9
cis-Platin	33.67	31.94–35.49

its anti-proliferative effect, lowering its efficacy of about 70% respect to compound **4** (indeed, the IC_{50} values of compound **2** and **4** are, respectively, 85.26 and 49.16 μM, Table 2). Moreover, the lack of activity of compound **1** could be ascribed to the lower lipophilicity respect to compound **2.** In a similar fashion, the higher lipophilic character of compound **4**, respect to the compound **3**, resulted in a higher anti-proliferative effect (IC_{50} 49.16 μM of compound **2** respect to 273.7 μM compound **3**, Table 2). In the compound **6** (IC_{50} 129.8 μM) the presence of another methoxy group at 2′ position of phenyl ring brings to a reduction in the antiproliferative activity respect to compound **4** and, once again, the lower lipophilicity of compound **5**, respect to **6**, completely abolishes the activity (Table 2). Concerning the compounds **7** and **8** that have one or two methoxy-naphthyl group(s) as substituent of the cyclopentadienyl respectively, the behavior is different. Indeed, the half-titanocene presents a higher activity than the corresponding titanocene (the IC_{50} values of compounds **7** and **8** are 63.13 μM and 171.8 μM, respectively).

In addition, a control experiment using non-malignant breast epithelial cells MCF-10A has been performed. As shown in Figure 4, the proliferative behavior of these cells is not affected by compound **2** but changed by compounds **4** and **6** only at the high dose of 40 μM; the compound **7** exerts a slight but statistically significant inhibitory effect at the doses of 5–10-20 μM, whereas *cis*-platin decreases cell growth at 20 and 40 μM. Moreover, the IC_{50} values of the most active compounds (i.e., **2**, **4**, **6** and **7**, Table 3) and of *cis*-platin have been calculated, evidencing that these new complexes have a more specific inhibitory effect on MCF-7 breast cancer cells, exhibiting a lesser antiproliferative activity than *cis*-platin on non-tumoral MCF-10A cells.

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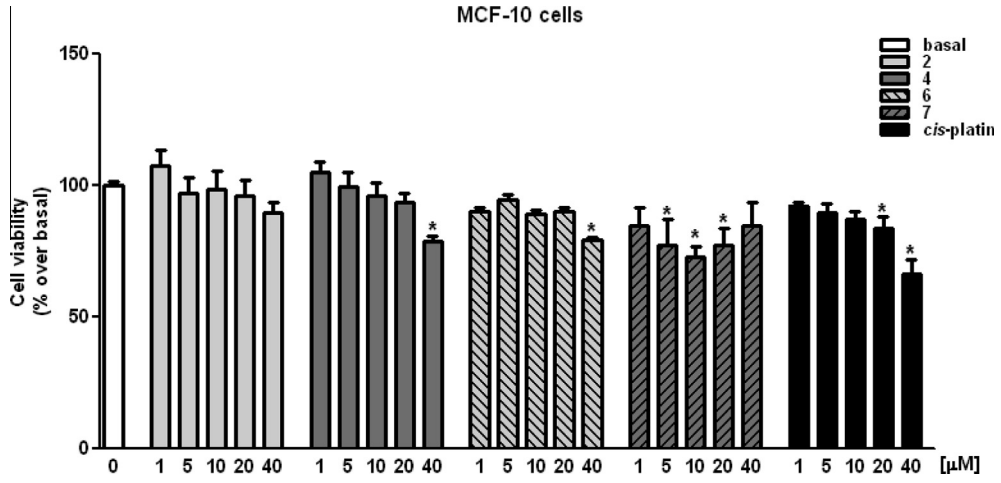

Figure 4. Effects of different doses of titanocene complexes on MCF-10A cell proliferation. Cells were treated for 72 h with the indicated concentrations of compounds **7**, **2**, **4**, **6** and *cis*-platin. Cell viability was evaluated by MTT assay. Graphs represent mean + SE of three independent experiments each performed in triplicate. Statistically significant differences are indicated (*, P <0.05 vs basal).

Table 3
IC_{50} of *cis*-platin and some titanocene complexes for MCF-10A cells

Compounds	IC_{50} (μM)	95% confidence intervals
2	395.0	286.0–545.5
4	181.7	159.5–207.1
6	145.4	129.3–163.5
7	90.59	67.95–120.8
cis-Platin	80.23	74.97–85.86

significant inhibitory effect at the doses of 5–10-20 μM, whereas *cis*-platin decreases cell growth at 20 and 40 μM. Moreover, the IC_{50} values of the most active compounds (i.e., **2**, **4**, **6** and **7**, Table 3) and of *cis*-platin have been calculated, evidencing that these new complexes have a more specific inhibitory effect on MCF-7 breast cancer cells, exhibiting a lesser antiproliferative activity than *cis*-platin on non-tumoral MCF-10A cells.

In order to verify if the decrease in cell growth was associated with nuclear morphological changes, we performed DAPI staining. This assay demonstrated that untreated MCF-7 cells had round nuclei with regular contours and large. After treatment with compound **4** cells showed nuclei with condensed DNA (Fig. 5A), a feature often associated with the onset of apoptosis, DNA damage but, as well, with other cellular processes.[33,34] We also investigated the status of the poly-(ADP)ribose polymerase (Parp-1),[35] which is a nuclear enzyme that catalyzes the transfer of the ADP-ribose moiety of NAD^+ to a specific subset of nuclear substrates in response to DNA damage.[36] It is an important regulator of the DNA base excision repair (BER) pathway and it is involved in the maintenance of genomic integrity and survival following genotoxic insults.[37–39]

In this paper, we revealed that treatment of compound **4** determines Parp-1 cleavage in the same way to *cis*-platin (Fig. 5B).

Thus, we next performed TUNEL assay in order to establish whether compound **4** was able to induce cell death by apoptosis; however, no clear evidence that treated cells were going under apoptosis have been found (data not shown).

Literature data reported that *cis*-platin is an inhibitors of DNA topoisomerases, which are essential enzymes involved in the regulation of the topological state of DNA during its replication, transcription, recombination and, as well, chromatin remodeling and are important for ensuing genomic integrity so that the possibility to interfere with these enzymes represents an effective strategy for cancer therapy.[40,41] Concerning this, DNA topoisomerases I and II are good targets of clinically significant classes of anticancer drugs being able to interfere with the enzyme–DNA complexes producing permanent DNA damages and triggering cell death.[42] Therefore, it would be desirable to synthesize compounds able to inhibit both the types, in order to minimize the onset of toxic side effects, due to the combinations of topo I and II poisons use and, eventually, drug resistance. The latter phenomenon is frequent when a selective inhibitor is used, probably due to the rise of the other isoforms in the cancer cellular context.[43] In order to examine whether these complexes, found to be able to exert antiproliferative effects, could act as topoisomerases poisons and, eventually, if they possess a dual activity on topoisomerases, we performed inhibition assays using human topoisomerases I and II. As shown in Figure 6A, the most active complexes (i.e., **2**, **4**, **6** and **7**, lanes d, e, f, and g respectively) have been found able to completely inhibit topoisomerase I activity, as *cis*-platin did, at the dose of 50 μM. Indeed super coiled (SC) DNA cannot be relaxed by the enzyme as, on the contrary, happens in the control reaction. Moreover, the same complexes **2**, **4**, **6** and **7** (lanes d, e, f, and g, respectively) are, as well, inhibitors of topoisomerase II activity, as indicated in Figure 6B, and this produces a fail of intact big-sized kinetoplast DNA (kDNA) migration on agarose gel. Similar results have been obtained using *cis*-platin as reference molecule (Fig. 6A, lane h), whereas in the control sample (vehicle) the enzyme is able to bind kDNA and release intact monomeric rings. The latter are visible at the bottom of gel as two DNA bands representing the nicked open circular minicircles and fully closed circular rings (decatenation products, Fig. 6B, lane c). In summary, the new synthesized complexes clearly block human topoisomerases I and II activity at the dose of 50 μM, as well as *cis*-platin. These results may well correlate with the chromatin condensation and parp-1 activation observed and discussed before, according to other published results.[44–46]

3. Conclusions

In this paper we reported the synthesis of new titanocene and half-titanocene complexes with a higher chemical stability to hydrolysis, low toxic effects on the proliferation of non-malignant breast cells and interesting antiproliferative effects on ER(+) breast cancer cells MCF-7, caused by a clear DNA topoisomerases inhibitory activity. Nevertheless further studies are needed to better define the molecular mechanism induced by these compounds that determine the breast cancer cell death, the presented outcomes open new interesting perspectives about the effectiveness in therapy of metal-based drugs.

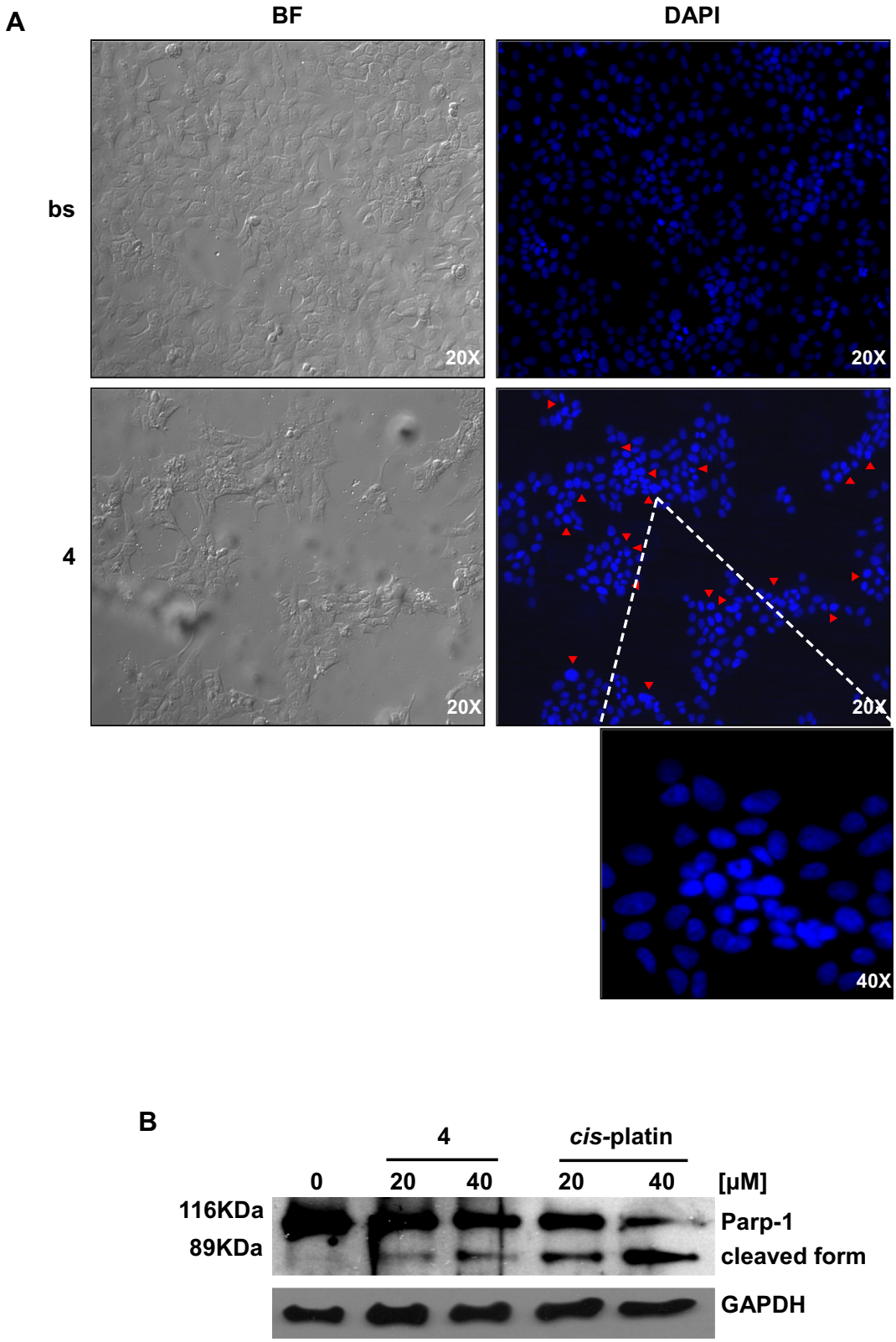

Figure 5. Effects of compound 4 treatment on MCF7 cell nuclei morphology and Parp-1 activation. MCF-7 cells were treated with compound **4** and *cis*-platin (20, 40 μM) for 72 h (A) or 24 h (B). (A) After treatment MCF7 cells were fixed with paraformaldehyde, dyed with DAPI and observed under fluorescent microscope (objective 20x or 40x). BF, brightfield; arrows indicate condensed nuclei. Images are from a representative experiment. (B) Western blot analyses of Parp-1 was performed on equal amounts of total proteins. Blots are representative of three independent experiments with similar results. GAPDH was used as a loading control.

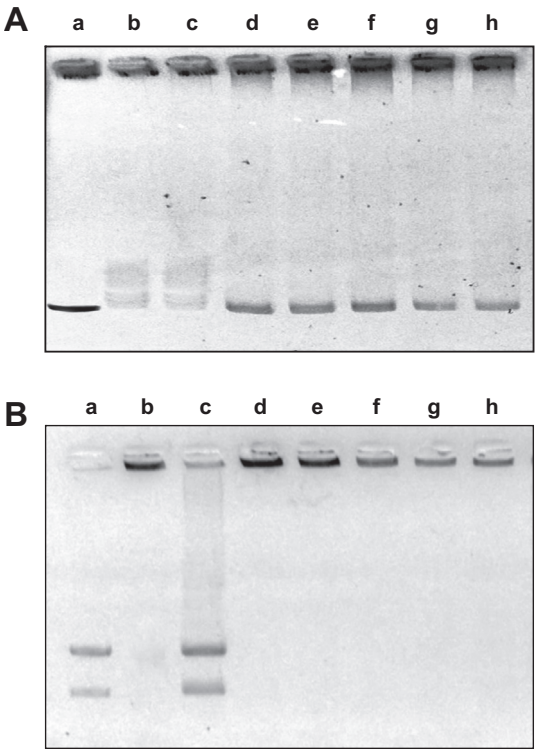

Figure 6. Evaluation of human topoisomerase I and II activity. (A): relaxation assay. Supercoiled DNA was incubated without or with human topoisomerase I in the absence or presence of the titanocene complexes or *cis*-platin at 50 μM: Lane a, super coiled DNA, lane b, relaxed DNA marker, lane c, vehicle (DMSO), lanes d, e, f, g and h, complexes **2**, **4**, **6**, **7** and *cis*-platin, respectively. (B): decatenation assay. kDNA was incubated without or with human topoisomerase II in the absence or presence of the titanocene complexes or *cis*-platin at 50 μM: Lane a, kDNA, lane b, decatenated DNA marker, lane c, linear kDNA, lane d, vehicle (DMSO), lanes d, e, f, g and h, complexes **2**, **4**, **6**, **7** and *cis*-platin, respectively.

4. Experimental section

4.1. Chemistry

The elemental analyses for C, H, N, were recorded on a ThermoFinnigan Flash EA 1112 series and performed according to standard microanalytical procedures. ^{1}H NMR, homodecoupled ^{1}H NMR, ^{1}H COSY and ^{13}C NMR spectra were recorded at 298 K on a Bruker Avance 300 Spectrometer operating at 300 MHz (^{1}H) and 75 MHz (^{13}C) and referred to internal tetramethylsilane. Molecular weights were determined by ESI mass spectrometry. ESI-MS analysis in positive and negative ion mode, were made using a Finnigan LCQ ion trap instrument, manufactured by Thermo Finnigan (San Jose, CA, USA), equipped with the Excalibur software for processing the data acquired. The sample was dissolved in acetonitrile and injected directly into the electrospray source, using a syringe pump, which maintains constant flow at 5 μl/min. The temperature of the capillary was set at 220 °C. All manipulations were carried out under oxygen- and moisture-free atmosphere in an MBraun MB 200 glove-box. All the solvents were thoroughly deoxygenated and dehydrated under argon by refluxing over suitable drying agents; while NMR deuterated solvents (Euriso-Top products) were kept in the dark over molecular sieves. $TiCl_4$, Titanium(IV) chloride tetrahydrofuran complex, Super Hydride ($LiBEt_3H$, 1.0 M solution in THF), and all chemicals were obtained from Aldrich chemical Co. and used without further purification. Cyclopentadiene was obtained by freshly cracked dicyclopentadiene. The four fulvenes and their relative lithium salt were prepared by following slightly modified reported procedures.

4.2. General synthesis

4.2.1. Synthesis of 6-methyl-6-aryl-fulvenes

The synthesis was carried out under nitrogen. Pyrrolidine (30.0 mmol) was added to a solution of suitable acetophenone derivatives or 6-methoxy-2-naphtaldehyde (20 mmol) and cyclopentadiene (50.0 mmol) in 30 ml of methanol. After addition, the color of the solution turned from colorless to red–orange. After 20 h, acetic acid (1.8 ml, 32.0 mmol) was added. The reaction mixture was diluted with 20 ml of a mixture of diethyl ether and water (1:1). The resultant organic layer was separated and the aqueous layer was washed with diethyl ether (3 × 20 ml). The combined organic extracts were washed with a saturated aqueous NaCl solution. The organic solution was dried over magnesium sulfate. The crude product was purified by column chromatography over silica gel and a mixture of *n*-hexane/ethyl acetate (9:1) as the eluent, obtaining a yield of between 22% and 35%.

4.2.1.1. Spectral data of 6-methyl-6-aryl fulvenes. 6-Methyl- 6-phenyl-fulvene [C_5H_4═CH—5′-$C_{10}H_6$—(OCH_3)]: ^{1}H NMR (δ ppm, $CDCl_3$): 7.39–7.29 (m, 5H, C_6H_5); 6.65–6.47 (m, 4H, C_5H_4); 2.54 (s, 3H, CH_3).

6-Methyl-6-(4′-methoxyphenyl)fulvene [C_5H_4═C(CH_3)—*p*-C_6H_4—OCH_3]: ^{1}H NMR (δ ppm, $CDCl_3$): 7.38–6.91 (m, 4H, C_6H_4); 6.63–6.23 (m, 4H, C_5H_4); 3.83 (s, 3H, OCH_3); 2.52 (s, 3H, CH_3).

6-Methyl-6-(2′,4′-dimethoxyphenyl)fulvene [C_5H_4═C(CH_3)—*2, 4*—C_6H_3—(OCH_3)$_2$] ^{1}H NMR (δ ppm, $CDCl_3$): 7.04–6.56 (m, 3H, C_6H_3); 6.47–6.06 (m, 4H, C_5H_4); 3.85 (s, 3H, OCH_3); 3.73 (s, 3H, OCH_3); 2.50 (s, 3H, CH_3).

6-(5′-Methoxynaphthyl)fulvene ^{1}H NMR (δ ppm, $CDCl_3$): 7.97–7.14 (m, 6H, $C_{10}H_6$); 7.34 (s, 1H, $C_{10}H_6$—C*H*—Cp); 6.69–6.36 (m, 4H, C_5H_4); 3.95 (s, 3H, OCH_3).

4.2.2. Synthesis of half-titanocene complexes (1, 3, 5, 7)

$LiBEt_3H$ (10 ml of a 1.0 M solution in THF) was concentrated by removal of the solvent by heating it to 60 °C under a vacuum of 10^{-2} mbar for 40 min and to 90 °C for other 20 min. The concentrated reagent was dissolved in diethyl ether (50 ml) and was transferred to a solution of 6-methyl-6-aryl-fulvene derivatives (5 mmol) in diethyl ether (60 ml). The solution was stirred (12 h), during which time the lithium cyclopentadienide intermediate precipitated from the solution and the color of the solution changed from red to yellow. After stirring, the precipitate was allowed to settle and was filtered to remove the filtrate. The white lithium salt was then collected on a frit and washed with diethyl ether (25 ml), dried briefly in vacuo and transferred to a Schlenk flask under nitrogen.

$TiCl_4$·2THF (0.4 mmol) was added to 20 ml of dry THF. The solution turned immediately from colourless to pale yellow. 0.4 mmol of the lithium cyclopenadienide intermediate were dissolved in 30 ml of THF dry and added dropwise to the solution containing the $TiCl_4$.The solution turned from yellow to dark red during addition. After this addition, the mixture was refluxed overnight and then cooled. The solvent was removed under reduced pression. The remaining residue was extracted with dichloromethane (30 ml) and filtered twice through celite to remove the LiCl. The black filtrate was washed twice with hexane (20 ml) and then dried under reduced pression to give a solid.

4.2.2.1. Spectral data of half-titanocene complexes. [(Cyclopentadienyl-ethyl-1-benzene)]-titanium-trichloride [C_5H_4-$CH(CH_3)$-C_6H_5] $TiCl_3$ (**1**): ^{1}H NMR (δ ppm, THF): 7.23–7.17 (5H, m, C_5H_4—$CH(CH_3)$—C_6H_5); 6.43–6.35 (4H, m, C_5H_4—$CH(CH_3)$—C_6H_5); 4.45 (1H, s, C_5H_4—$CH(CH_3)$—C_6H_5); 1.58 (3H, s, C_5H_4—$CH(CH_3)$—$C_6H_4(OCH_3)$). ^{13}C NMR (δ ppm, THF): 23.9 (C_5H_4—$CH(CH_3)$—$C_6H_4(OCH_3)$), 43.6 (C_5H_4—$CH(CH_3)$—C_6H_5), 109.2, 114.8, 120.1, 127.5, 127.8, 138.8 (C_5H_4—$CH(CH_3)$—C_6H_5).

Mass (E.I., 70 eV, *m*/*z*): 286 [L-Ti-Cl_2]$^{\cdot+}$. Anal. Calcd for $C_{13}H_{13}Cl_3Ti$: C, 48.27; H, 4.05. Found: C, 48.10; H, 3.95.

[(Cyclopentadienyl-ethyl-1-(4-methoxy-benzene)]-titanium-trichloride [C_5H_4—CH(CH_3)(*p*-C_6H_4—OCH_3)]$TiCl_3$ (**3**): ^{1}H NMR (δ ppm, C_6D_6): 7.04–6.37 (m, 4H, C_5H_4—CH(CH_3)—C_6H_4(OCH_3)); 5.75–5.45 (m, 4H, C_5H_4—CH(CH_3)—C_6H_4(OCH_3)); 4.63 (s, 1H, C_5H_4—*CH*(CH_3)—C_6H_4(OCH_3)); 3.30 (s, 3H, C_5H_4—CH(CH_3)—C_6H_4(*OCH*$_3$)); 1.58 (s, 3H, C_5H_4—CH(*CH*$_3$)—C_6H_4(OCH_3)). ^{13}C NMR (δ ppm, C_6D_6): 23.2 [C_5H_4—CH(*CH*$_3$)—C_6H_4(OCH_3)], 40.6 [C_5H_4—*CH*(CH_3)—C_6H_4(OCH_3)], 55.0 [C_5H_4—CH(CH_3)—C_6H_4(*OCH*$_3$)], 114.2, 114.5, 120.0, 120.8, 122.0, 123.3, 129.5, 138.8, 159.1 [*C*$_5$*H*$_4$—CH(CH_3)—*C*$_6$*H*$_4$(OCH_3)]. Mass (E.I., 70 eV, *m*/*z*): 317 [L-Ti-Cl_2]$^{\cdot+}$. Anal. Calcd for $C_{14}H_{15}Cl_3OTi$: C, 47.57; H, 4.28. Found: C, 47.72; H, 3.98.

[(Cyclopentadienyl-ethyl-1-(2,4-dimethoxy-benzene)]-titanium-trichloride [C_5H_4—CH(CH_3)(2,4-C_6H_3—(OCH_3)$_2$)]$TiCl_3$ (**5**): ^{1}H NMR (δ ppm, C_6D_6): 6.91–5.92 (m, 7H, *C*$_5$*H*$_4$—CH(CH_3)—*C*$_6$*H*$_3$(OCH_3)$_2$); 5.06 (s, 1H, C_5H_4—*CH*(CH_3)—C_6H_3(OCH_3)$_2$); 3.22, 3.23 (2s, 6H, C_5H_4—CH(CH_3)—C_6H_3(*OCH*$_3$)$_2$); 1.70 (s, 3H, C_5H_4—CH(*CH*$_3$)—C_6H_3(OCH_3)$_2$). ^{13}C NMR (δ ppm, C_6D_6): 23.7 [C_5H_4—CH(*CH*$_3$)—C_6H_3(OCH_3)$_2$], 30.5 [C_5H_4—*CH*(CH_3)—C_6H_3(OCH_3)$_2$], 54.3, 55.2 [C_5H_4—CH(CH_3)—C_6H_3(*OCH*$_3$)$_2$], 98.6, 103.7, 114.5, 120.8, 121.4, 131.4, 136.4, 150.2, 160.1 [*C*$_5$*H*$_4$—CH(CH_3)—*C*$_6$*H*$_3$(OCH_3)$_2$]. Mass (E.I., 70 eV, *m*/*z*): 347 [L-Ti-Cl_2]$^{\cdot+}$. Anal. Calcd for $C_{15}H_{17}Cl_3O_2Ti$: C, 46.98; H, 4.47. Found: C, 47.01; H, 4.26.

[(Cyclopentadienyl)-methylene-(5-methoxy-naphtalene)]-titanium-trichloride [$C_5H_4CH_2$($C_{10}H_6$—OCH_3)]$TiCl_3$ (**7**): ^{1}H NMR (δ ppm, THF): 7.66–7.07 (m, 6H, *C*$_{10}$*H*$_6$); 6.46–6.38 (m, 4H, *C*$_5$*H*$_4$); 4.19 (s, 2H, $C_{10}H_6$—*CH*$_2$—Cp); 3.85 (s, 3H, O*CH*$_3$). ^{13}C NMR (δ ppm CD_2Cl_2): 35.1 ($C_{10}H_6$—*CH*$_2$—Cp), 55.9 (OCH_3), 104.9, 124.1, 125.4, 128.1, 128.7, 129.3, 132.5, 147.0, 155.9 (*C*$_5$*H*$_4$, *C*$_{10}$*H*$_6$). Mass (E.I., 70 eV, *m*/*z*): 353 [L-Ti-Cl_2]$^{\cdot+}$. Anal. Calcd for $C_{17}H_{15}Cl_3OTi$: C, 52.42; H, 3.88. Found: C, 52.78; H, 3.65.

4.2.3. Synthesis of titanocene complexes (2, 4, 6, 8)

The lithium intermediate was obtained as described before for the synthesis of half-titanocenes.

$TiCl_4$·2THF (0.28 mmol) was added to 20 ml of dry THF. The solution turned immediately from colourless to pale yellow. 0.56 mmol of the lithium cyclopentadienide intermediate were dissolved in 30 ml of THF dry and added dropwise to the solution containing the $TiCl_4$.The solution turned from yellow to dark red during addition. After this addition, the mixture was refluxed overnight and then cooled. The solvent was removed under reduced pression. The remaining residue was extracted with dichloromethane (30 ml) and filtered twice through celite to remove the LiCl. The black filtrate was washed twice with hexane (20 ml) and then dried under reduced pression to give a dark red solid.

4.2.3.1. Spectral data of titanocene complexes. Bis[(cyclopentadienyl-ethyl-1-benzene)]-titanium-dichloride [$C_5H_4CH(CH_3)C_6H_5$]$_2$ $TiCl_2$ (**2**): ^{1}H NMR (δ ppm, THF): 7.23–7.16 (m, 10H, C_5H_4—CH(CH_3)—*C*$_6$*H*$_5$); 6.40–6.35 (m, 8H, *C*$_5$*H*$_4$—CH(CH_3)—C_6H_5)); 4.44 (s, 2H, C_5H_4—*CH*(CH_3)—C_6H_5); 1.58 (s, 6H, C_5H_4—CH(*CH*$_3$)—C_6H_4(OCH_3)). ^{13}C NMR (δ ppm, THF): 22.9 [C_5H_4—CH(*CH*$_3$)—C_6H_4(OCH_3)], 43.4 [C_5H_4—*CH*(CH_3)—C_6H_5], 110.2, 115.1, 120.4, 127.0, 127.8, 139.1 [*C*$_5$*H*$_4$—CH(CH_3)—*C*$_6$*H*$_5$]. Mass (E.I., 70 eV, *m*/*z*): 384 [L_2-Ti]$^{\cdot+}$. Anal. Calcd for $C_{26}H_{26}Cl_2Ti$: C, 68.28; H, 5.73. Found: C, 68.62; H, 5.32.

Bis-[(cyclopentadienyl-ethyl-1-(4-methoxy-benzene)]-titanium-dichloride [$C_5H_4CH(CH_3)$(*p*C_6H_4—OCH_3)]$_2TiCl_2$ (**4**): ^{1}H NMR (δ ppm, C_6D_6): 7.04–6.37 (m, 8H, C_5H_4—CH(CH_3)—*C*$_6$*H*$_4$(OCH_3)); 5.81–5.44 (m, 8H, *C*$_5$*H*$_4$—CH(CH_3)—C_6H_4(OCH_3)); 4.60 (s, 2H, C_5H_4—*CH*(CH_3)—C_6H_4(OCH_3)); 3.28 (s, 6H, C_5H_4—CH(CH_3)—C_6H_4(*OCH*$_3$)); 1.58 (s, 6H, C_5H_4—CH(*CH*$_3$)—C_6H_4(OCH_3)). ^{13}C NMR (δ ppm, C_6D_6): 21.9 [C_5H_4—CH(*CH*$_3$)—C_6H_4(OCH_3)], 40.2 [C_5H_4—*CH*(CH_3)—C_6H_4(OCH_3)], 54.6 [C_5H_4—CH(CH_3)—C_6H_4(*OCH*$_3$)], 113.8, 114.2, 115.7, 119.6, 120.3, 129.1, 138.3, 142.7, 158.7 [*C*$_5$*H*$_4$—CH(CH_3)—*C*$_6$*H*$_4$(OCH_3)]. Mass (E.I., 70 eV, *m*/*z*): 479 [L_2-Ti-Cl]$^{\cdot+}$. Anal. Calcd for $C_{28}H_{30}Cl_2O_2Ti$: C, 65.01; H, 5.85. Found: C, 65.32; H, 5.42.

Bis-[(cyclopentadienyl-ethyl-1-(2,4-dimethoxy-benzene)]-titanium-dichloride [$C_5H_4CH(CH_3)$(2,4-C_6H_3(OCH_3)$_2$]$_2TiCl_2$ (**6**): ^{1}H NMR (δ ppm, C_6D_6): 6.88–6.28 (m, 6H, C_5H_4—CH(CH_3)—*C*$_6$*H*$_3$(OCH_3)$_2$]; 5.92–5.70 (m, 8H, *C*$_5$*H*$_4$—CH(CH_3)—C_6H_3(OCH_3)$_2$); 5.07 (2H, C_5H_4—*CH*(CH_3)—C_6H_3(OCH_3)$_2$); 3.32, 3.20 [2s, 12H, C_5H_4—CH(CH_3)—C_6H_3(*OCH*$_3$)$_2$]; 1.70 (s, 6H, C_5H_4—CH(*CH*$_3$)—C_6H_3(OCH_3)$_2$). ^{13}C NMR (δ ppm, C_6D_6): 23.3 [C_5H_4—CH(*CH*$_3$)—C_6H_3(OCH_3)$_2$], 38.8 [C_5H_4—*CH*(CH_3)—C_6H_3(OCH_3)$_2$], 52.1, 53.2 [C_5H_4—CH(CH_3)—C_6H_3(*OCH*$_3$)$_2$], 98.4, 103.5, 115.8, 121.3, 122.0, 136.3, 135.5, 153.0, 159.6 [*C*$_5$*H*$_4$—CH(CH_3)—*C*$_6$*H*$_3$(OCH_3)$_2$]. Mass (E.I., 70 eV, *m*/*z*): 504 [L_2-Ti]$^{\cdot+}$. Anal. Calcd for $C_{30}H_{34}Cl_2O_4Ti$: C, 62.41; H, 5.94. Found: C, 62.59; H, 5.59.

Bis-[(cyclopentadienyl)-methylene-(5-methoxy-naphtalene)]-titanium-dichloride [C_5H_4—CH_2—($C_{10}H_6$—OCH_3)]$_2TiCl_2$ (**8**): ^{1}H NMR (δ ppm, CD_2Cl_2): 7.68–7.01 (m, 12H, *C*$_{10}$*H*$_6$); 6.38–6.35 (m, 8H, *C*$_5$*H*$_4$); 3.90 (s, 4H, $C_{10}H_6$—*CH*$_2$—Cp); 3.88 (s, 6H, O*CH*$_3$). ^{13}C NMR (δ ppm, CD_2Cl_2): 34 ($C_{10}H_6$—*CH*$_2$—Cp), 55.2 (O*CH*$_3$), 105.4, 124.3, 125.2, 128.0, 128.8, 129.4, 132.8, 146.8, 156.1 (*C*$_5$*H*$_4$, *C*$_{10}$*H*$_6$). Mass (E.I., 70 eV, *m*/*z*): 516 [L_2-Ti]$^{\cdot+}$. Anal. Calcd for $C_{34}H_{30}Cl_2O_2Ti$: C, 69.29; H, 5.13. Found: C, 69.58; H, 4.89.

4.3. Cell cultures and treatments

MCF-7 breast cancer cells (an ER positive human breast cancer cells, obtained from American Type Culture Collection (ATCC), Manassas, VA, USA) were maintained as previously described.[47] MCF-10A human mammary epithelial cells, obtained from American Type Culture Collection (ATCC, Manassas, VA, USA), were maintained in DMEM-F12 supplemented with 10% horse serum (HS), 1% glutamine, 1% penicillin/streptomycin, 0.5 mg/ml hydrocortisone, 20 ng/ml hEGF (human epidermal growth factor) and 0.1 mg/ml cholera enterotoxin (Sigma–Aldrich, Milano, Italy) (complete medium). Cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 and were screened periodically for Mycoplasma contamination. All titanocene complexes were dissolved in dimethylsulfoxide (DMSO) (Sigma, St. Louis, Missouri, USA) at a concentration of 10 mM and diluted in DMEM/F12 medium supplemented with 1% DCC-FBS (dextran-coated charcoal-treated newborn calf serum serum) to obtain the working concentration.

4.4. Assessment of cell viability

MCF-7 and MCF-10A cells were seeded on twenty-four well plates (0.2×10^5 cells/well) and grown for 48 h in complete medium. Before being treated, cells were starved in DMEM/F12 serum free medium for 24 h to the purpose of cell cycle synchronization. The effect of the different doses of different titanocene complexes was measured using the MTT assay as previously described.[32,47–50] Seventy two hours after treatments, fresh MTT (Sigma), re-suspended in PBS, was added to each well (final concentration (0.33 mg/mL). After 2 h incubation, cells were lysed with 1 mL of DMSO. Each experiment was performed in triplicate and the optical density was measured at 570 nm in a spectrophotometer. Each experiment was performed in triplicate and the optical density was measured at 570 nm in a spectrophotometer. Results are represented as percent (%) of basal.

4.5. Western Blot analysis

Twenty μg of protein were subjected to western blot analysis.[51] Blots were incubated overnight at 4 °C with antibodies against Parp-1(from Santa Cruz Biotechnology, Santa Cruz CA, USA).

Membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Amersham Pharmacia Biotech, Piscataway, NJ) and immunoreactive bands were visualized with the ECL western blotting detection system (Amersham Pharmacia Biotech, Piscataway, NJ). To assure equal loading of proteins, membranes were stripped and incubated overnight with Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (Santa Cruz Biotechnology).

4.6. Determination of nuclear morphological changes

Cells were grown on glass coverslips, and then treated in FBS-DCC medium for 24 h. Cells were washed with PBS and fixed in 4% formaldehyde for 10 min at room temperature. Fixed cells were washed with PBS and incubated with 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) (0.2 μg/mL) for 10 min in a humidified chamber, protected from light, at 37 °C. Cells were then washed three times with cold PBS and one drop of mounting solution was added. Cell nuclei were observed and imaged under an inverted fluorescence microscope (200× magnification) with excitation at 350 nm and emission at 460 nm.

4.7. Human topoisomerase I relaxation assay

Relaxation assays have been performed in a final volume of 20 μL and assembled as follows. 0.25 μg of supercoiled pHOT1 in TE buffer [TE: 10 mM Tris–HCl (pH 7.5), 1 mM EDTA] (TopoGEN) has been added to a solution containing water (variable volume) and 1× assay buffer (10 mM Tris–HCl (pH 7.9), 1 mM EDTA, 0.5 mM NaCl, 0.1% bovine serum albumin, 0.1 mM spermidine and 5% glycerol). Next, the compounds to be tested have been added and the reaction initiated by addition of recombinant human topo I (2 U) (TopoGEN). The reactions were incubated at 37 °C for 30 min and terminated by the addition of 5 × stop buffer (5% sarkosyl, 25% glycerol, 0.125% bromophenol blue). Samples underwent to Proteinase K digestion (50 μg/mL) at 37 °C for 30 min, followed by extraction with an equal volume of chloroform/isoamylic alcohol (24:1), vortexed and centrifuged for 30 s. The upper aqueous phase has been withdrawn, loaded onto a 1% agarose gel containing 1X TAE buffer (diluted from 50× buffer containing 242 g Tris base, 57.1 ml glacial acetic acid and 100 ml of 0.5 M EDTA) without ethidium bromide (EB). After the run, agarose gel has been colored with 1× TAE buffer containing EB (0.5 μg/ml) for 30 min and destained with distilled water for 15 min, then visualized using a UV transilluminator.

4.8. Human Topoisomerase II decatenation assay

Decatenation assays have been performed in a final volume of 20 μL, as follows. 0.3 μg of kDNA (topoGEN) have been added to a solution containing water, 1× assay buffer [which contains: 50 mM Tris–HCl, pH of 8, 150 mM NaCl, 10 mM $MgCl_2$, 0.5 mM Dithiothreitol (DTT) and 30 μg/mL bovine serum albumin (BSA)], and 1 mM ATP. The compounds to be tested have been added and the reaction started by the addition of 3 U of human topoisomerase II (topoGEN) and incubated at 37 °C for 30 min. At the end, 5× stop buffer has been added and the samples have been treated as described in the previous paragraph. The aqueous phase has been loaded on a 1% agarose gel containing 1× TAE buffer with EB (0.5 μg/ml) and visualized using an UV transilluminator.

4.9. Data analysis and statistical methods

All experiments were performed at least three times. Data were expressed as mean values ± standard deviation (SD), statistical significance between control (basal) and treated samples were analyzed using GraphPad Prism 5.0 (GraphPad Software, Inc.; La Jolla, CA) software. Basal (untreated cells) and treated cells were compared using the analysis of variance (ANOVA) with Kruskal–Wallace test and post hoc Dunn's Multiple Comparison Test. Significance was defined as $p < 0.05$.

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