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Synthesis, characterization and cytotoxic activity on breast cancer cells of new half-titanocene derivatives

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ABSTRACT

A series of novel titanocene-complexes has been prepared and evaluated for their growth regulatory effects in MCF7 and SkBr3 breast cancer cells. The capability of some of these compound to elicit relevant repressive effects on cancer cell growth could be taken into account towards novel pharmacological approaches in cancer therapy.

Platinum based anticancer drugs like *cis*-platin, carboplatin and oxaliplatin are commonly used in the treatment of a wide number of tumors, including lung, colorectal, ovarian, head and neck, as well as genitourinary, and breast cancers. However, the efficacy of platinum-based drugs is often compromised because of the substantial risk for severe toxicities, including neurotoxicity.[1]

Over the last few years a lot of research has developed novel metal-based anticancer drugs, with the aim of improving clinical effectiveness, reducing general toxicity and broadening the spectrum of activity.[2]

The search for novel metal-based antitumor drugs, other than Pt agents, includes the investigation of the cytotoxic activity of copper(I/II)[2] and titanocene[3] compounds.

Particular attention has been recently devoted to new copper(I) complexes. Some of these were tested for their cytotoxic properties against several human tumor cell lines, such as HL60 (promyelocytic leukemia), MCF7 (breast cancer), HCT-15 (colon cancer), HeLa (cervix cancer), A549 (lung cancer), 2008 (ovarian cancer sensitive to *cis*-platin) and C13* (ovarian cancer resistant to *cis*-platin).[2]

A great deal of research has been focused on a variety of transition metal complexes bearing labile *cis* chlorides or similar ligands. Among the candidate drugs, the pseudotetrahedral metallocene complexes of the type $(C_5H_5)_2MCl_2$ represent a seemingly logical extension of *cis*-platin derivatives and have received much attention.[4] These complexes are made up of a metal core consisting of transition metals as Ti, Nb, Mo, etc. The coordination sites of the metal are occupied by two cyclopentadienyl rings (C_5H_5 or Cp) and two labile ligands (i.e., Cl). Köpf-Maier and Köpf[5] have investigated the antitumor activities of a whole series of metallocene dichloride complexes (varying the transition metal) in vivo. From this research, titanocene dichloride (TDC) (Fig. 1) exhibited the most promising chemotherapeutic activity among all other metallocenes tested.[6]

Consequently, numerous analogues of TDC were developed and well studied, such as titanocene Y (Fig. 1).

In particular, the anti-proliferative activity of titanocene Y and other titanocenes has been studied in 36 human tumor cell lines.[7] In vitro and ex vivo experiments showed that renal cancer is a major target for this novel class of titanocenes, although they showed a significant activity also against ovarian, prostate, cervix, lung, colon and breast tumors.[8]

Recently, we reported the synthesis and the cytotoxic activity of some titanocene derivatives obtained replacing the aryl-methoxylic group on cyclopentadienyl of titanocene Y with the ethenyl-methoxy group, in order to have a stronger electron donor effect on the cationic species responsible for the cytotoxic activity. We also verified the influence of leaving ligands on the activity by substituting chlorine atoms with dimethylamide, oxalate or aminoacid groups.[3] It is worth noting that in Ref. 3, the highest cytotoxic activity was reported for half-titanocene complex $\mathbf{T}_1$,

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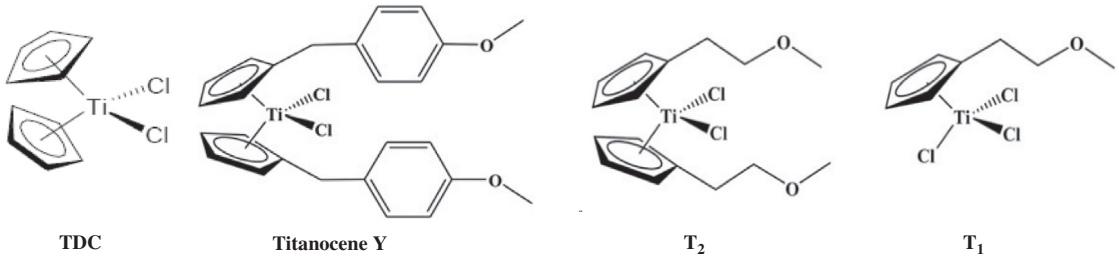


Figure 1. Titanocene dichloride (TDC); bis-[(*p*-methoxybenzyl)cyclopentadienyl]titanium dichloride (titanocene Y); bis-cyclopentadienyl-ethenylmethoxyl-titanium dichloride $\mathbf{T_2}$ and cyclopentadienyl-ethenylmethoxyltitanium trichloride $\mathbf{T_1}$.

compared to that of titanocene $\mathbf{T_2}$, titanocene Y and *cis*-platinum. Some of the synthesized compounds showed a good cytotoxicity, in particular complexes bis-cyclopentadienyl-ethenylmethoxyl-titanium dichloride $\mathbf{T_2}$ and cyclopentadienyl-ethenylmethoxyl-titanium trichloride $\mathbf{T_1}$ gave values very similar to *cis*-platin on MCF-7 cell lines, with the IC_{50} comparable to the ones reported for titanocene Y. Moreover, the half-titanocene complex ($\mathbf{T_1}$) also showed a good cytotoxic activity, comparable to that of *cis*-platin, on HEK-293 cells. The results of hydrolysis of our titanocenes showed unequivocally that the leaving groups (Cl, $N(CH_3)_2$, C_2O_4 or glycine) significantly affect even hydrolysis rate of cyclopentadienyl groups, being chloride and oxalate more stable.[3]

Thus, the presence of substituents, aryl methoxy group on cyclopentadienyl ring in titanocene Y or ethenyl-methoxy group in titanocene $\mathbf{T_2}$ or in the half-titanocene $\mathbf{T_1}$, produce compounds with interesting cytotoxic activity. Although generalizations regarding structure–activity relationships are not yet clear, we could hypothesize that the neutral nucleophilic substituents of cyclopentadienyl (aryl methoxy or ethenyl-methoxy group) could intramolecularly coordinate 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 to the metal center.[9–11]

As mentioned above, several examples of titanocene-complexes showing cytotoxic activity were reported, but to the best of our knowledge the cyclopentadienyl-ethenylmethoxyl-titanium trichloride represents the first example of half-titanocene complex with interesting cytotoxic activity.

Therefore, the aim of this study was the synthesis and the characterization of some half-titanocenes compounds (see Fig. 2) by nuclear magnetic resonance (NMR), mass spectroscopy and elemental analysis. All these complexes contain different substituents on the Cp ligands, able to stabilize the titanium cation by intramolecular coordination. Preliminary cytotoxic studies of these titanium based compounds have been carried out, as well.

Compound **5a** was synthesized in order to verify if the activity was higher for half-titanocene Y than titanocene Y, as it was for the bis-cyclopentadienyl-ethenylmethoxyl-titanium dichloride $\mathbf{T_2}$ and cyclopentadienyl-ethenylmethoxyl-titanium trichloride $\mathbf{T_1}$. Compounds **5b**, **5e** and **5f** bear in different positions methoxyl groups, which may make ligands much more coordinating, except for the methoxyl in position 4. Compound **5d** has no substituents on the aryl, but the phenyl is of course able to coordinate to titanium. Finally, the dimethylamino group in position 4 of the aryl moiety of **5c** has a strong capabilities to bond metal-cation.

The synthesis of complexes was carried out according to Scheme 1. The syntheses of proligands fulvene **3a–d** and **3f** were carried out as outlined in references,[12–15] whereas **3e** (2,4-dimethoxyphenyl) fulvene was synthesized in good yield according to literature method[14,16] starting from 2,4-dimethoxy benzaldehyde.

The lithium salt of the ligand was obtained by reacting the suitable fulvene with Super Hydride ($LiBEt_3H$) in dry diethyl ether. Then, it was isolated and subsequently reacted with 1 equiv of $TiCl_4{\cdot}2THF$ in dry THF. The reaction product was purified following common procedures and isolated in high yield (see Scheme 1). Elemental analysis (C, H, N) was in accordance with the proposed formulation. ^1H COSY experiments allowed the assignment of all the proton resonances of the ^1H NMR spectrum, whereas DEPT experiments were useful for the attribution of ^{13}C NMR signals. The synthesized half-titanocenes were also characterized by mass spectrometry. The mass spectra show the molecular ion and the fragmentation of the complexes (i.e., ligand, [ligandTi]).[17] These data allowed us to have an unambiguous structural determination, as reported in Scheme 1.

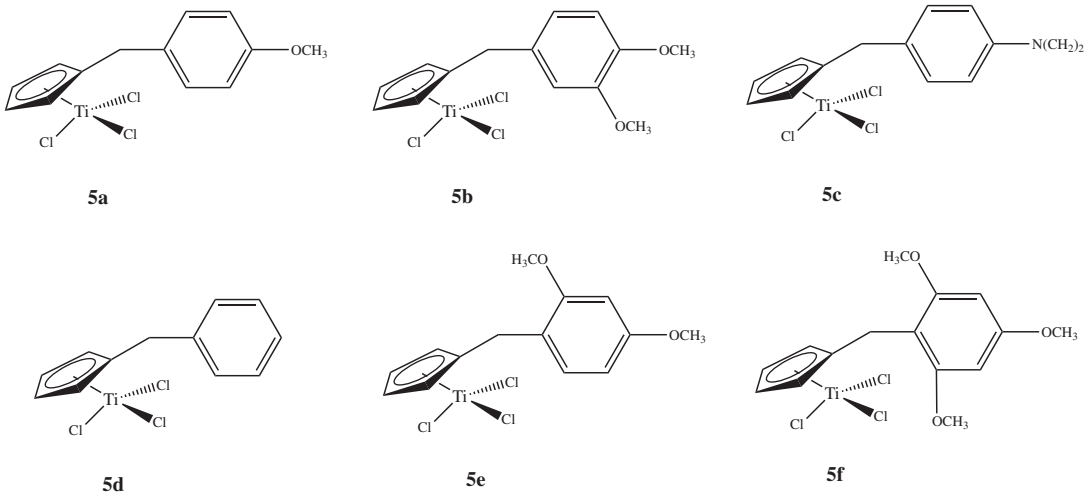
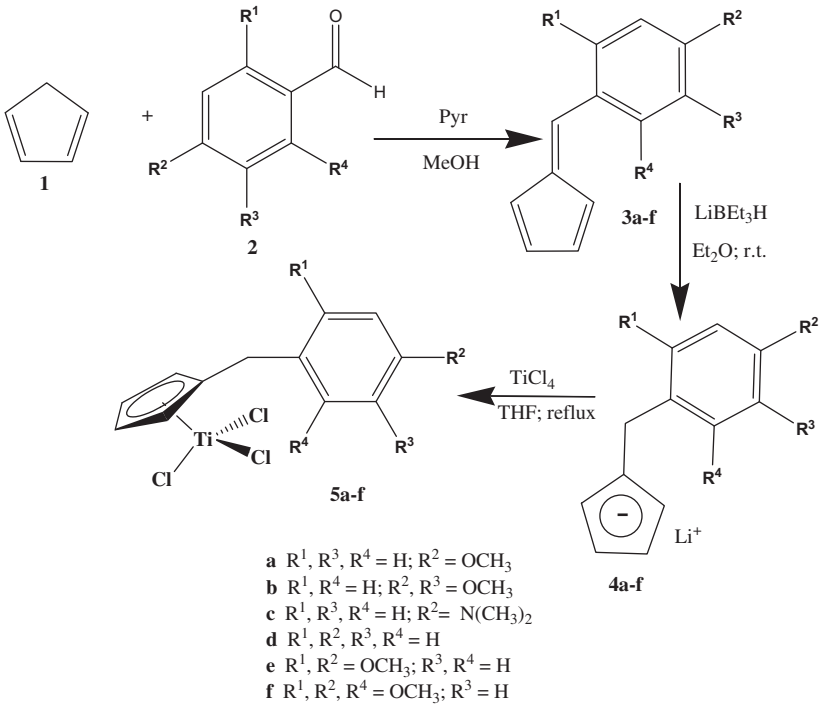


Figure 2. Structures of synthesized complexes.



Scheme 1. Synthetic route for the preparation of ligands and half-titanocene complexes **5a–f**.

Table 1
Hydrolysis results of **5a–f** complexes in DMSO/D_2O solution at rt followed by ^1H NMR

Complex	% Cp ring hydrolysis			
	5 min	4 h	8 h	24 h
5a	<1	<1	<1	<5
5b	<1	<1	<1	<5
5c	<1	<1	<5	17
5d	9	24	40	41
5e	<1	<1	<1	<5
5f	<1	<1	<1	<5

Hydrolysis stability of the six half-titanocene complexes (**5a–f**) has been determined in aqueous solution, 90% DMSO by ^1H NMR spectroscopy, in order to correlate the chemical stability and coordination chemistry of these complexes with their observed cytotoxic activity. Since we can expect that rapid hydrolysis of leaving group (–Cl) and cyclopentadienyl ligands could give way to biologically inactive species, active species could be generate if the Cp rings remain metal bound.

Hydrolysis of aromatic rings of **5a–f** was evaluated by integration of two signals of protons of cyclopentadienyl bonded to metal, as to newly formed multiplet of substituted cyclopentadiene. Table 1 reports the results of our hydrolysis tests.

The complexes that show the highest hydrolytic stability are **5a**, **5b**, **5e** and **5f**. In particular, the cyclopentadienyl rings of complexes are hydrolyzed only for less than 5% after 24 h, whereas complexes **5c** and **5d** are hydrolyzed for 17% and 41%, respectively (Table 1).

These data provide sufficient evidence that the presence of coordinating groups on the aryl substituent of the cyclopentadienyl are effective for the stabilization of the complexes. Therefore, these coordinating groups might be fundamental to increase, if active, their biological effectiveness.

In order to investigate the effects on cancer cell proliferation of the novel synthesized compounds, we treated for 5 days MCF7 and SkBr3 breast cancer cells with each compound.[18–20] Cells were also exposed to *cis*-platin in order to compare the anticancer effects of the complexes to this well-known chemotherapeutic. It should be noted that by using the compounds mentioned above, SkBr3 cells

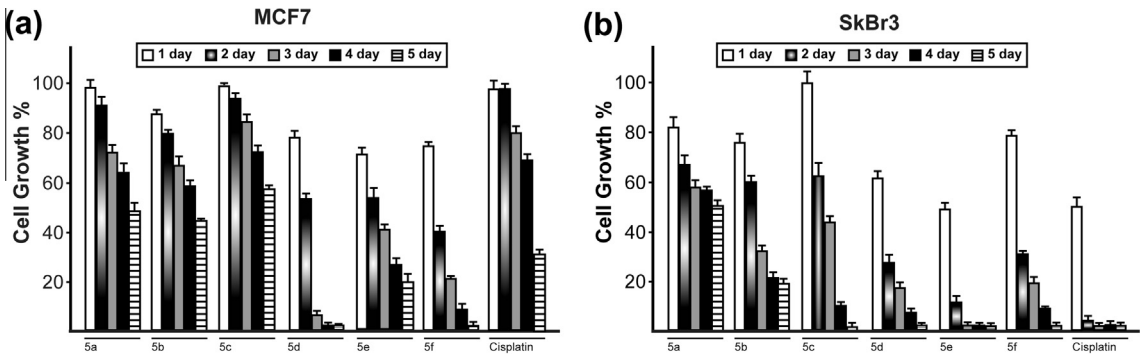


Figure 3. Evaluation of growth responses to 10 μM of **5a–f** in MCF7 (a) and SkBr3 (b) breast cancer cells, as determined by using the MTT assay. Cell viability was expressed as the percentage of cells treated with the different compounds respect to cells treated with vehicle. Cells ($5–8 \times 10^4$ ml^{-1}) were treated for 1 day up to 5 days, as indicated.

resulted to be more responsive to the treatment compared to MCF7 cells. Among all tested compounds, **5d**, **5e** and **5f** elicited repressive effects on the proliferation of both cell lines (Fig. 3). In particular, **5d** strongly decreased the viability of MCF7 cells after 3 days of treatment, whereas **5e** showed the highest antitumor activity on SkBr3 cells after 2 days, being the most active compound on this cell line (Fig. 3). In particular, **5d**, **5e** and **5f** showed a strongest cytotoxic effect on MCF7 than *cis*-platin. Moreover **5e** showed a similar cytotoxic activity on SkBr3 compared to *cis*-platin.

In conclusion, a series of novel titanocene-complexes has been synthesized and evaluated for their growth regulatory effects in MCF7 and SkBr3 breast cancer cells. Among these compounds, that showed moderate to high antitumor activity, the strongest antiproliferative activity against MCF7 cells was displayed especially by **5d**, whereas **5e** elicited relevant repressive effects on SkBr3 cells. Hence, the capability of these compounds to elicit inhibitory effects on cancer cell growth could be taken into account towards novel pharmacological approaches in cancer therapy. Therefore further experiments would be helpful to investigate the molecular mechanism involved.

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17. *Experimental section*: 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 six fulvenes and their relative lithium salt were prepared by following the reported procedures.[14,16]
Synthesis of half-titanocene complexes ***5a,b***: Lithium cyclopentadienide intermediate (1.83 mmol) was dissolved in dry THF (20 ml) to give a colourless solution. $TiCl_4$ (18.30 mmol) was added at 0 °C to give a dark red solution. This was refluxed overnight and then cooled. The solvent was removed under reduced pression. The remaining residue was extracted with dichlorometane (30 ml) and filtered through celite to remove the LiCl. The filtrate was washed twice with hexane (20 ml) and then dried under reduced pression to give a solid.
Spectral data of newly synthesized compounds: [(4-Methoxybenzyl) cyclopentadienyl]-titanium-trichloride [C_5H_4–CH_2–C_6H_4–OCH_3]$TiCl_3$ (**5a**). Black solid. ^{1}H NMR (δ ppm, CD_2Cl_2, 300 MHz): 3.78 [s, 3H, C_5H_4–CH_2–C_6H_4–*OCH_3*], 4.01 [s, 2H, C_5H_4–*CH_2*–C_6H_4–OCH_3], 6.80 [m, 4H, *C_5H_4*–CH_2–C_6H_4–OCH_3], 6.83–7.01 [d, 4H, C_5H_4–CH_2–*C_6H_4*–OCH_3]. ^{13}C NMR (δ ppm, CD_2Cl_2, 75 MHz): 55.90 [C_5H_4–CH_2–C_6H_4–*OCH_3*], 45.0 [C_5H_4–*CH_2*–C_6H_4–OCH_3], 114.0–128.70–130.0–132.0–135.90–147.80–158.60 [*C_5H_4*–CH_2–*C_6H_4*–OCH_3]. Mass (E.I., 70 eV, *m/z*): 273 [L-TiCl-Li]$^{\cdot+}$, 186 [L]$^{\cdot+}$. Calcd for $C_{13}H_{13}Cl_3OTi$ (%): C, 46.00; H, 3.86. Found (%): C, 46.21; H, 3.84.
[(3,4-Di-methoxybenzyl)-cyclopentadienyl]-titanium-trichloride [C_5H_4–CH_2–C_6H_3–$(OCH_3)_2$]$TiCl_3$ (**5b**). Brown solid. ^{1}H NMR (δ ppm, THF-d_8, 300 MHz): 3.73 [s, 6H, C_5H_4–CH_2–C_6H_3–$(OCH_3)_2$], 3.98 [s, 2H, C_5H_4–*CH_2*–C_6H_3–$(OCH_3)_2$], 6.29–6.42 [m, 4H,*C_5H_4*–CH_2–C_6H_3–$(OCH_3)_2$], 6.71–6.76 [d, 2H, *C_6H_3*–$(OCH_3)_2$], 6.84 [s, 1H, *C_6H_3*–$(OCH_3)_2$]. ^{13}C NMR (δ ppm, CD_2Cl_2, 75 MHz): 55.50 [C_5H_4–CH_2–C_6H_3–*$(OCH_3)_2$*], 36.80 [C_5H_4–*CH_2*–C_6H_3–$(OCH_3)_2$], 112.20–113, 30–115.40–120.80–122.50–132.80–137.10–149.20–150.30 [*C_5H_4*–CH_2–*C_6H_3*–$(OCH_3)_2$]. Mass (E.I., 70 eV, *m/z*): 286 [L-Ti-Na]$^{\cdot+}$. Calcd for $C_{14}H_{15}Cl_3O_2Ti$ (%): C, 45.51; H, 4.09. Found (%): C, 45.91; H, 4.04.
(4-(*N*,*N*-Dimethylbenzanilido)-cyclopentadienyl-titanium-trichloride [C_5H_4–CH_2–C_6H_4–$N(CH_3)_2$]$TiCl_3$ (**5c**). Red solid. ^{1}H NMR (δ ppm, THF, 300 MHz): 2.98 [s, 6H, C_5H_4–CH_2–C_6H_4–*$N(CH_3)_2$*], 3.89 [s, 2H, C_5H_4–*CH_2*–C_6H_4–$N(CH_3)_2$], 6.45–6.67 [m, 4H, *C_5H_4*–CH_2–C_6H_4–$N(CH_3)_2$], 7.10–7.31 [d, 4H, C_5H_4–CH_2–*C_6H_4*–$N(CH_3)_2$]. ^{13}C NMR (δ ppm, THF-d_8, 75 MHz): 41.70 [C_5H_4–CH_2–C_6H_4–*$N(CH_3)_2$*], 33.10 [C_5H_4–*CH_2*–C_6H_4–$N(CH_3)_2$], 115.90–117.60–119.20–128.70–130.70–136.0–137.10 [*C_5H_4*–CH_2–*C_6H_4*–$N(CH_3)_2$]. Mass (E.I., 70 eV, *m/z*): 371 [L-$TiCl_3$-Na]$^{\cdot+}$. Calcd for $C_{14}H_{16}Cl_3NTi$ (%): C, 47.70; H, 4.57. Found (%): C, 47.92; H, 4.17; N, 3.79.
(Benzyl)-cyclopentadienyl-titanium-trichloride [C_5H_4–CH_2–C_6H_5]$TiCl_3$ (**5d**). Red solid. ^{1}H NMR (δ ppm, THF-d_8, 300 MHz): 4.06 [s, 2H, C_5H_4–*CH_2*–C_6H_5], 6.33–6.45 [m, 4H, *C_5H_4*–CH_2–C_6H_5], 7.22–7.24 [m, 5H, C_5H_4–CH_2–*C_6H_5*]. ^{13}C NMR (δ ppm, THF-d_8 75 MHz): 36.70 [C_5H_4–*CH_2*–C_6H_5], 110.90–115.10–122.60–128.0–128.60–140.60 [*C_5H_4*–CH_2–*C_6H_5*]. Mass (E.I., 70 eV, *m/z*): 242 [L-Ti-K]$^{\cdot+}$, 163 [L-Li]$^{\cdot+}$. Calcd for $C_{12}H_{11}Cl_3Ti$ (%): C, 46.58; H, 3.58. Found (%): C, 46.17; H, 3.32.
(2,4-Di-methoxybenzyl)-cyclopentadienyl-titanium-trichloride [C_5H_4–CH_2–C_6H_3–$(OCH_3)_2$]$TiCl_3$ (**5e**). Brown solid. ^{1}H NMR (δ ppm, THF-d_8, 300 MHz): 3.72–3.760 [s, 6H, C_5H_4–CH_2–C_6H_3–*$(OCH_3)_2$*], 3.90 [s, 2H, C_5H_4–*CH_2*–C_6H_3–$(OCH_3)_2$], 6.28–6.29 [m, 4H, *C_5H_4*–CH_2–C_6H_3–$(OCH_3)_2$], 6.40–6.46 [d, 2H,C_5H_4–CH_2–*C_6H_3*–$(OCH_3)_2$] 7.01 [s, 1H, C_5H_4–CH_2–*C_6H_3*–$(OCH_3)_2$]. ^{13}C NMR (δ ppm, CD_2Cl_2, 75 MHz): 55.40–55.50 [C_5H_4–CH_2–C_6H_3–*$(OCH_3)_2$*], 31.80 [C_5H_4–*CH_2*–C_6H_3–$(OCH_3)_2$], 99.10–104.80–116.60–121.90–123.30–131.40–137.60–159.20–161.0 [*C_5H_4*–CH_2–*C_6H_3*–$(OCH_3)_2$]. Mass (E.I., 70 eV, *m/z*): 286 [L-Ti-Na]$^{\cdot+}$. Calcd for $C_{14}H_{15}Cl_3O_2Ti$ (%): C, 45.51; H, 4.09. Found (%): C, 45.91; H, 4.04.
(2,4,6-Tri-methoxybenzyl)-cyclopentadienyl-titanium-trichloride [C_5H_4–CH_2–C_6H_2–$(OCH_3)_3$]$TiCl_3$ (**5f**). Black solid. ^{1}H NMR (δ ppm, THF, 300 MHz): 3.72–3.77 [s, 9H, C_5H_4–CH_2–C_6H_2–*$(OCH_3)_3$*], 3.90 [s, 2H, C_5H_4–*CH_2*–C_6H_2–$(OCH_3)_3$], 6.30–6.34 [m, 4H, *C_5H_4*–CH_2–C_6H_2–$(OCH_3)_3$], 6.47 [s, 2H, C_5H_4–CH_2–*C_6H_2*–$(OCH_3)_3$]. ^{13}C NMR (δ ppm, THF-d_8, 75 MHz): 54.70 [C_5H_4–CH_2–C_6H_2–*$(OCH_3)_3$*], 31.05 [C_5H_4–*CH_2*–C_6H_2–$(OCH_3)_3$], 98.40–104.0–115.80–122.50–130.60–137.70–158.30–160.30 [*C_5H_4*–CH_2–*C_6H_2*–$(OCH_3)_3$]. Mass (E.I., 70 eV, *m/z*): 317 [L-Ti-Na]$^{\cdot+}$. Calcd for $C_{15}H_{17}Cl_3O_3Ti$ (%): C, 45.09; H, 4.29. Found (%): C, 45.39; H, 4.24.
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19. *Cell culture*: MCF7 breast cancer cells were maintained in DMEM/F-12 supplemented with 10% fetal bovine serum (FBS), 100 mg/ml penicillin/streptomycin and 2 mM L-glutamine (Invitrogen, Gibco, Milan, Italy). SkBr3 breast cancer cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 mg/ml penicillin/streptomycin and 2 mM L-glutamine (Invitrogen, Gibco, Milan, Italy). Cells were switched to medium without serum the day before experiments and thereafter treated in medium supplemented with 1% FBS.
Inhibition of cell proliferation: The effects of each compound on cell viability were determined with the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay,[20–23] which is based on the conversion of MTT to MTT-formazan by mitochondrial enzyme. Cells were seeded in quadruplicate in 96-well plates in regular growth medium and grown until 70–80% confluence. Cells were washed once they had attached and then treated

with 10 μM each compound for indicated time (for 1 day up to 5 days). Relative cell viability was determined by MTT assay according to the manufacturer's protocol (Sigma–Aldrich, Milan, Italy). Mean absorbance for each drug dose was expressed as a percentage of the control untreated well absorbance and plotted versus drug concentration. IC_{50} values represent the drug concentrations that reduced the mean absorbance at 570 nm to 50% of those in the untreated control wells.

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