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Original article

Synthesis, characterization and cytotoxicity studies of methoxy alkyl substituted metallocenes

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ABSTRACT

Five titanocene derivatives and one zirconium analogous, having cyclopentadienylethenylmethoxy ligand, were synthesized and fully characterized by NMR, FT-IR, and elemental analysis. Two of these complexes showed a good cytotoxic activity on human breast cancer (MCF-7) cell lines. Moreover, the half-titanocene disclosed also a good cytotoxic activity on human embryonic kidney (HEK-293). Additionally, a study on the rate of hydrolysis of these compounds showed that the leaving groups significantly affect the rate of hydrolysis of cyclopentadienyl groups too. The different activity of synthesized compounds was tentatively related to the rate of hydrolysis.

1. Introduction

Despite the discovery of *cis*-platin in the treatment of cancer [1–4], there has been a considerable exploration on the antitumoral activity of other transition metal complexes. For instance, recently the attention has been focused on titanium based complexes, which could have significant potential effect against solid tumor. Titanocene dichloride (Cp_2TiCl_2) shows an average antiproliferative activity *in vitro* but promising results *in vivo* [5,6]. Cp_2TiCl_2 reached Phase II clinical trials, but its efficiency in patients with metastatic renal cell carcinoma [7] or metastatic breast cancer [8] was too low to be pursued. The mechanism and biological action of titanocene dichloride seem to be different from that of *cis*-platin.

In fact, since titanocene dichloride does not strongly bond to nucleotides and nucleobases, Sadler et al. proposed, on the basis of model studies, that a titanium species could complex to phosphate and give a mechanism whereby titanium is delivered to DNA. The titanium(IV) ion forms a strong complex with the transferrin, a human plasma protein, which has been involved in the transport and delivery of titanium ions to cancer cells, by binding to specific iron(III) binding sites [9].

Considerable work has been performed in developing therapeutic analogues of Cp_2TiCl_2 by varying the central metal, the labile ligands (Cl) and the bis-cyclopentadienyl moiety. In particular, small changes to the Cp ligand can strongly affect the hydrolytic stability and water solubility properties of the metallocenes and have an impact on the cytotoxic activity. Most of the analyzed titanocene complexes have polar substituents on cyclopentadienyl ring such as alkoxo, amino or electron-withdrawing groups as carboxylic acid and esters which have demonstrated, in some cases, a very high activity in antitumoral tests [10–17].

Several attempts have been made to improve the aqueous solubility of Cp_2TiCl_2 through the appendage of polar side chains to the Cp ligands. A number of alkylammonium hydrochloride moieties or cationic, water-soluble derivatives of Cp_2TiCl_2 have been prepared and showed good activity on several cell lines [14,18]. Furthermore, when the chloride ligands were replaced with glycine, water-soluble complexes with high stability at physiological pH were obtained. They were observed to form stable complexes with nucleotides at pH 7, although further biological studies were not reported [19].

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Recently, a novel method starting from fulvenes [20–23] and other precursors allowed direct access to highly substituted *ansa*-titanocenes [24–27], containing a carbon–carbon bridge, revealing promising activity [28,29].

A large number of unbridged titanocene analogues containing aromatic groups attached to the Cp ligand have also been synthesized [30]. One of the most promising drugs of this series was bis-[(p-methoxybenzyl)cyclopentadienyl]titanium dichloride (titanocene Y) depicted in Fig. 1, and its antiproliferative activity has been studied in 36 human tumor cell [31] and in explanted human tumors [32–35]. These *in vitro* and *ex vivo* experiments showed that prostate, cervix and renal cancer cells are prime targets for these novel classes of titanocenes. Moreover, titanocene Y has been tested on breast cancer cell line MCF-7 and a promising medium-high cytotoxic activity with IC_{50} values of 76 μM results, close enough to that of *cis*-platin (37 μM) [34].

The oxalate complex obtained from titanocene Y, by a simple anion-exchange reaction, having a substantially less labile chelating ligand in replacement of the chlorine groups, was reported to have 13-fold increase in activity in relation to titanocene Y during *in vitro* studies against the LLC-PK cell line. Differences in the IC_{50} values can reflect the greater hydrolytic stability of the oxalate derivative [36]. It is worth noting that this effect was not observed with all the titanocene derivatives studied [37].

Sweeney et al. reported the synthesis of some titanocenes having different heteroaryl substituents (furyl, thiophenyl or N-methyl-pyrrole) in order to investigate the effects of heteroaryl substituted titanocene on cytotoxicity. The results were compared with those of benzyl substituted titanocenes, obtaining a structure-activity relationship [38].

Despite the significant studies on titanocene derivatives, not much research has been carried out on similar zirconium based compounds. Allen et al. reported the cytotoxic activity of functionalized water-soluble zirconocene derivatives against a range of human tumor cell lines, showing an interesting cytotoxic behavior, very similar to those reported for titanium analogous [39].

The current efforts in the chemistry of group 4 metallocenes are focused on the design of new compounds with different substituents which may increase their cytotoxicity.

In this paper we report the synthesis and the characterization by nuclear magnetic resonance (NMR), FT-IR spectroscopy and elemental analysis of titanocenes compounds containing dialkyl ether groups appended to the Cp ligands, having different leaving groups on the metal. Moreover, a homologous zirconium compound was also synthesized. We have studied the hydrolytic behavior of synthesized complexes and finally, their cytotoxic activities were compared to the *cis*-platin *in vitro* on human breast cancer (MCF-7), human embryonic kidney (HEK-293) and murine macrophage (J774.A1) cell lines.

2. Results and discussion

The aim of this work was the synthesis, the characterization and the cytotoxicity studies of novel titanocene complexes by making appropriate changes to titanocene Y. We have replaced 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 have verified the influence of leaving ligands on the activity by substitution of chlorine atoms with dimethylamide, oxalate or aminoacid groups. Moreover, di-(ethenylmethoxy-cyclopentadienyl)-zirconium-dichloride was synthesized, in order to compare its activity with titanium analogous, and ethenylmethoxy-cyclopentadienyl-half-titanocene, with the purpose of evaluating the effect of lower steric hindrance compared to titanocene derivatives. All the synthesized compounds are reported in Fig. 2.

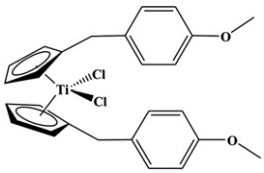


Fig. 1. Bis-[(p-methoxybenzyl)cyclopentadienyl]titanium dichloride (titanocene Y).

2.1. Chemistry

2.1.1. Synthesis of $[C_5H_4–CH_2CH_2OCH_3]_2TiCl_2$ (**2**)

The synthesis of this product was carried out in good yields by reaction of the lithium salt of the ligand $Li[C_5H_4–CH_2CH_2OCH_3]$ with $TiCl_4$.

The lithium–cyclopentadienide-ethenyl-2-methoxyl was obtained as reported in literature [40] and shown in Scheme 1. Reaction of sodium-cyclopentadienyde with 2-chloro-1-methoxyethane in THF at low temperature produced 2-cyclopentadienylethenyl-methyl-ether (**1**). The product was purified by distillation and characterized by NMR analysis.

After the dissolution of 2-cyclopentadienylethenyl-methyl-ether in THF dry, lithium butyl was added to form *in situ* the corresponding lithium salt, which subsequently reacted with half equivalent of titanium-tetrachloride. The reaction product was purified following common procedures and isolated in high yield. Elemental analysis (C, H, N) agreed with the proposed formulation. ^1H COSY experiments allowed the assignment of all the proton resonances of the ^1H NMR spectrum, whereas HSQC and DEPT experiments were useful for the attribution of ^{13}C NMR signals (see Experimental part).

2.1.2. Synthesis of $[C_5H_4–CH_2CH_2OCH_3]TiCl_3$ (**3**)

The synthesis of this product was carried out in the same way as compound **2**, by the reaction between 1 equiv of $TiCl_4$ and the lithium salt of the ligand, making small changes to the one previously reported in the literature [41]. The characterization of **3** was obtained as usual by NMR analysis (see Experimental part).

2.1.3. Synthesis of $[C_5H_4–CH_2CH_2OCH_3]_2Ti[N(CH_3)_2]_2$ (**4**)

The synthesis of this product was carried out by the reaction of the potassium salt of the ligand $K[C_5H_4–CH_2CH_2OCH_3]$ with half equivalent of $Ti[N(CH_3)_2]_4$ (see Scheme 1). The reaction product was purified following common procedures and isolated in good yield. In Experimental part are reported the full attribution of ^1H and ^{13}C NMR resonances.

2.1.4. Synthesis of $[C_5H_4–CH_2CH_2OCH_3]_2Ti(C_2O_4)$ (**5**)

The synthesis of bis-cyclopentadienide-ethenyl-2-methoxyl-titanium-oxalate was performed by reaction of **2** with silver oxalate in THF dry (see Scheme 1). After the filtration of silver-chloride, the reaction product was purified as usual and isolated in good yield. The characterization of the complex was completely performed by the attribution of the resonances of ^1H and ^{13}C NMR spectra (see Experimental part).

2.1.5. Synthesis of $[C_5H_4–CH_2CH_2OCH_3]_2Ti(gly)_2$ (**6**)

The synthesis of bis-cyclopentadienide-ethenyl-2-methoxyl-titanium-bis-glycine was carried out by the reaction of **2** with 2 equiv of glycine in methanol containing 1% of water (see Scheme 1). The characterization of **6** was performed by the complete attribution of the signals in the ^1H and ^{13}C NMR spectra (see Experimental part).

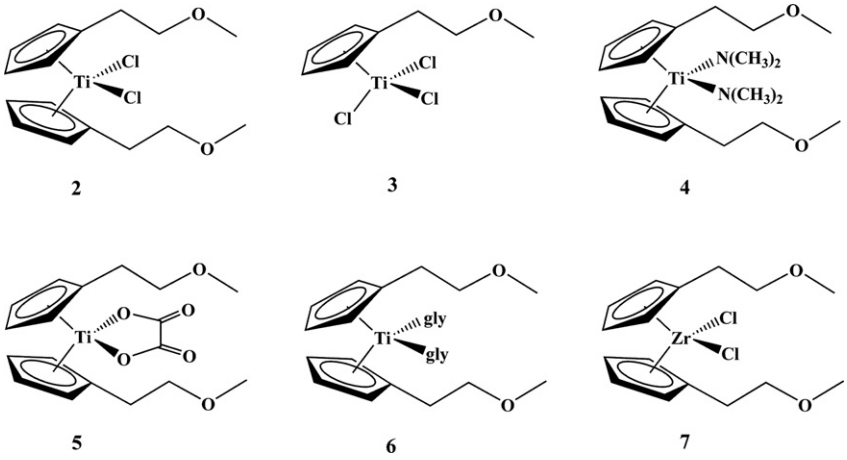


Fig. 2. Sketches of the synthesized complexes.

*2.1.6. Synthesis of $[C_5H_4{-}CH_2CH_2OCH_3]_2ZrCl_2$ (**7**)*

The synthesis of this product was very similar to **2**, and was performed by the reaction of the lithium salt of the ligand Li $[C_5H_4{-}CH_2CH_2OCH_3]$ with half equivalent of $ZrCl_4$ (see Scheme 1). The reaction product was purified as usual and isolated in good yield. NMR analysis allowed the characterization of the product (see Experimental part).

Elemental analysis (C, H, N) of all the synthesized compounds is in agreement with the proposed formulations, and ^1H and ^{13}C NMR analysis on the basis of COSY, DEPT and HSQC experiments has allowed the determination of their structure in solution in a very satisfactory way. In Fig. 3 the ^1H and ^{13}C NMR spectra of complex **2** are reported as an example.

FT-IR spectra of complexes **2**–**7** confirm the coordination environment of the metals, in fact band attributable to C–O–C stretching, for complexes **2**, **3**, **4**, **5**, **6** and **7** are at 1118, 1113, 1121, 1105, 1116 and 1108 cm^{-1}, respectively, whereas it is at 1150 cm^{-1} in the free ligand. The carbon–carbon stretching of cyclopentadienyl rings are at 1641, 1639, 1660, 1636, 1640 and 1635 cm^{-1}, respectively for complexes **2**, **3**, **4**, **5**, **6** and **7** whereas it is at 1670 cm^{-1} in the free ligand. It is worth noting that the stretching of the N–C bond in complex **4** is at 3370 cm^{-1} whereas it is at 3380 cm^{-1} in the $Ti[N(CH_3)_2]_4$, and as for C=O in the complex **5** it is at 1719 cm^{-1} whereas it is at 1730 cm^{-1} in $Ag_2C_2O_4$ reagent.

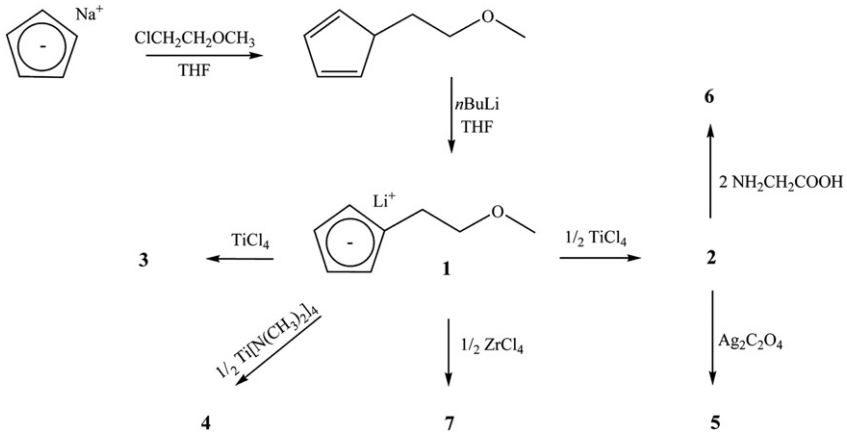
2.2. Hydrolysis tests

Hydrolysis stabilities of group 4 metallocenes **2**–**7** have been determined in aqueous solution – 10% DMSO and in 100% DMSO, by ^1H NMR spectroscopy, in order to correlate the chemical stability and coordination chemistry of these complexes, with their observed cytotoxic activities. Since the rapid hydrolysis of the leaving group (–Cl, $-N(CH_3)_2$, oxalate or glycine) and cyclopentadienyl ligands could probably give biologically inactive species, an active species could be generated if the cyclopentadienyl rings remain bound to the metal.

The hydrolysis of aromatic rings was evaluated through the integration of protons signals of methylene connected to the cyclopentadienyl bonded to metal at 2.7 ppm, with respect to the newly formed multiplet of substituted cyclopentadiene at 2.9 ppm. In Fig. 4 the spectra of the products of the hydrolysis of complex **2** in DMSO, at different times, are reported.

In Table 1 the effect of the solvent on rates of hydrolysis of cyclopentadienyl rings are reported.

The products which show the highest hydrolytic stability are **2**, **3** and **5**. In particular, in DMSO the cyclopentadienyl rings of complex **3** after 24 h are hydrolyzed only for 21%, whereas complexes **2** and **5** are hydrolyzed for 53 and 82%, respectively. It is worth noting that, only 5% of the same complexes are hydrolyzed in DMSO/D_2O.



Scheme 1. Synthetic route for the preparation of ligand **1** and metal complexes **2**–**7**.

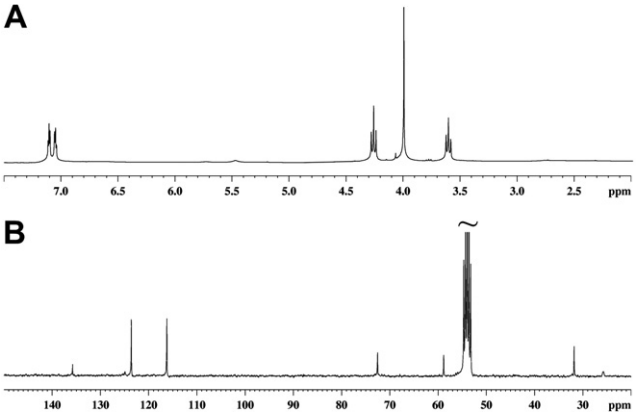


Fig. 3. ^{1}H (A) and ^{13}C NMR (B) spectra of complex **2**. TMS scale.

Complexes **4**, **6** and **7** already after 5 min are almost completely hydrolyzed in both the tested solvent.

Starting from the reflection that the different activities of the complexes could be related to their different stabilities, the hydrolysis stability represents a first possible indication on the achievable cytotoxic effects of synthesized compounds. Thus, the leaving ligands influence the stability to hydrolysis of the complex in the solvents used for cytotoxic tests, since they are bonded to the metal with different strength, so that some of these remain coordinated when placed in biological fluids, preventing the formation of active species of the complex, namely the cationic species, moreover they affect the solubility of the complex in the solvents used for cytotoxicity assays.

2.3. Pharmacology

Table 2 reports IC_{50} values for ligand **1**, complexes **2**–**7** and *cis*-platin on human breast cancer (MCF-7), human embryonic kidney (HEK-293) and murine macrophage (J774.A1) cell lines.

As reference drug we chose to use *cis*-platin for the only purpose of testing the susceptibility of our cell lines to cytotoxicity. More interesting data are obtained for products **1**, **2** and **3** on MCF-7 cell lines. In fact, MTT assays revealed that these compounds showed an IC_{50} value very similar to the *cis*-platin's one. It is interesting to point out that these compounds showed a cytotoxic activity comparable to the one reported for titanocene Y (76 μM) [35].

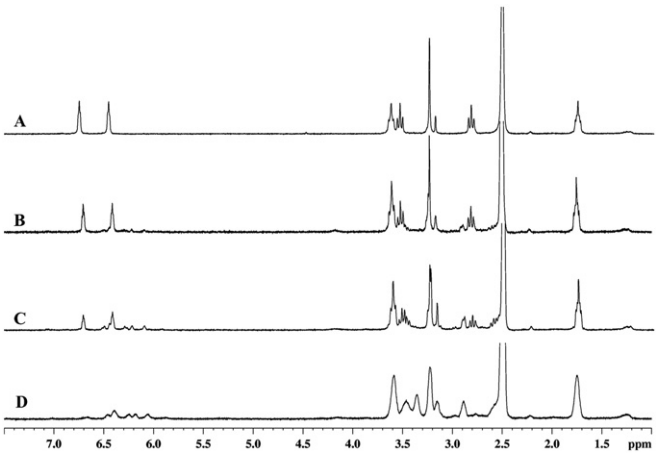


Fig. 4. ^{1}H NMR spectra of $[C_5H_4{-}CH_2CH_2OCH_3]_2TiCl_2$ at 37 °C in [D6]DMSO (A) 5 min after the dissolution, (B) 4.5 h after the dissolution, (C) 8.5 h after the dissolution and (D) 24 h after the dissolution.

Table 1
Hydrolysis results of **2**–**7** complexes in DMSO and DMSO/D_2O solution at 37 °C followed by ^{1}H NMR.

Compounds	Reaction time (h)	% Cp′ hydrolysis	
		DMSO	DMSO/D_2O 1/9
2	0.08	<1	<1
	4.50	15	<1
	8.50	18	<1
	24.0	53	<5
3	0.08	<1	<1
	4.50	4	<1
	8.50	6	<1
	24.0	21	5
4	0.08	>99	>99
	4.50	>99	>99
	8.50	>99	>99
	24.0	>99	>99
5	0.08	<1	<1
	4.50	18	<1
	8.50	42	<1
	24.0	82	<5
6	0.08	>99	>99
	4.50	>99	>99
	8.50	>99	>99
	24.0	>99	>99
7	0.08	63	>99
	4.50	>99	>99
	8.50	>99	>99
	24.0	>99	>99

Moreover, complex **3** showed a good cytotoxic activity, similar to the *cis*-platin's on HEK-293. It is worth noting that complexes **4** and **7** were active only on J774.A1 cell line. Further studies are needed to better evaluate the differences observed between the cell lines used.

Complexes **2** and **7** differ in the metal ligand. In fact complex **2**, that is more cytotoxic on MCF-7 cells, presents titanium, while complex **7** presents zirconium. We can hypothesize that the presence of titanium could be responsible for cytotoxic activity. In fact complex **2** on MCF-7 cells displays an activity comparable to the ones obtained by titanocene Y and *cis*-platin, while the cytotoxicity of complex **7** was not detectable. Complex **3**, which has only one cyclopentadienyl ring coordinated, so less steric encumbrance, with more electronic unsaturation, and having three leaving groups, showed a good cytotoxic activity on two cell lines (*i.e.* MCF-7 and HEK-293).

However, considering the low number of synthesized molecules it is not possible to make a structure–activity relationship.

Table 2
Cytotoxic behavior of synthesized compounds.

Compounds	Cell line		
	MCF-7	HEK-293	J774.A1
1	65	>100	>100
2	84	>100	>100
3	57	64	>100
4	>100	>100	95
5	>100	>100	>100
6	n.d.	n.d.	n.d.
7	>100	>100	67
cis-Platin	37	67	>100

The cytotoxic activity of compounds was evaluated as IC_{50} (μM): the concentration of compound that affords cell growth by 50% as compared to control on the following cell lines: MCF-7 human breast cancer cells, HEK-293 human embryonic kidney cells, J774.A1 murine macrophage cells. The *cis*-platin was used as standard drug.

3. Conclusions

In this work a cyclopentadienyl derivative has been synthesized and used to prepare six group 4 metal complexes. All the compounds were fully characterized by NMR, FT-IR and elemental analysis. Some of synthesized compounds have a good cytotoxicity, in particular either ligand **1** and complexes **2** and **3** show on MCF-7 cell lines IC_{50} values very similar to that of *cis*-platin, and comparable to that reported for titanocene Y. Moreover, complex **3** (half-titanocene) showed also a good cytotoxic activity, comparable to that of found in *cis*-platin on HEK-293. This could be due to both its low rate of hydrolysis and its lower steric encumbrance with respect to titanocene compounds.

Moreover, the results of the hydrolysis of compounds **2**–**7** show unequivocally that the leaving groups (Cl, $N(CH_3)_2$, C_2O_4 or glycine) significantly affect even the hydrolysis rate of cyclopentadienyl groups. This is probably because the reaction of hydrolysis in the case of compounds **4** and **6** should produce dimethylamino (see (a) in the following reaction) and glycine chlorydrate (see (b) in the following reaction), respectively:

(a) $Cp'_2Ti[N(CH_3)_2]_2 + H_2O \rightarrow [Cp'_2Ti(H_2O)(OH)]^+[OH]^- + HN(CH_3)_2$

(b) $Cp'_2Ti[OCOCH_2NH_2]_2 + H_2O \rightarrow [Cp'_2Ti(H_2O)(OH)]^+[OH]^- + HOOCCH_2NH_3^+Cl^-$

Thus, in one case we would have a basic and in the other an acid environment and both could promote the hydrolysis reaction of cyclopentadienyl.

Furthermore, it is worth noting that the atomic radius of the metal plays a crucial role in the cytotoxic activity of compounds, in fact the complex **7**, which is very similar to complex **2** (they have the same basic structure, differing only in the type of metal cation: in complex **2** it is titanium and in the complex **7** it is zirconium) does not show cytotoxic activity on cell line MCF-7, whereas it was active on J774.A1. Compound **2** has an interesting activity on cell line MCF-7, whereas it was not active on J774.A1.

4. Experimental section

4.1. Spectroscopic measurements

The elemental analyses for C, H, N, Cl were recorded on a ThermoFinnigan Flash EA 1112 series and were performed according to standard microanalytical procedures. ^1H NMR, homodecoupled ^1H NMR, ^1H COSY, ^1H NOESY, HSQC and ^{13}C $\{^1H\}$ NMR spectra were recorded at 298 K on a Bruker Avance 300 spectrometer operating at 300 MHz (^1H) and 75 MHz (^{13}C) and referred to internal tetramethylsilane. Fourier transform infrared (FTIR) spectra were obtained at a resolution of 2.0 cm^{-1} with a Bruker-Vector 22 FTIR spectrometer equipped with a deuterated triglycine sulphate (DTGS) detector and a Ge/KBr beam splitter. The frequency scale was internally calibrated to 0.01 cm^{-1} using a He–Ne reference laser. Thirty-two scans were signal-averaged to reduce spectral noise.

4.2. Chemistry

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. The anhydrous compounds $ZrCl_4$, $TiCl_4$, $Ti[N(CH_3)_2]$ (Strem, Aldrich) were used as received. Potassium hydride and lithium butyl were purchased from Aldrich. The neutral ligand $[C_5H_5–CH_2CH_2OCH_3]$ was prepared by following the reported procedure [40]. The half-titanocene $[C_5H_4–CH_2CH_2OCH_3]TiCl_3$ **3** was synthesized as reported in literature [41].

4.2.1. Synthesis of $[C_5H_4–CH_2CH_2OCH_3]_2TiCl_2$ (**2**)

To a solution of neutral ligand (1.0 g, 8.1 mmol) in THF dry (40 mL), a stoichiometic amount of *n*-BuLi (2.5 M solution in hexane, 3.5 mL) was slowly added at −78 °C. The solution was warmed up to room temperature and left stirred overnight, obtaining a yellow lithium intermediate. Afterward the solution was treated at −78 °C with 0.446 mL (4.06 mmol) of $TiCl_4$ and stirred overnight and then it was filtered to remove LiCl. The solvent was evaporated at reduced pressure and the red-brown solid dried in vacuum. The yield was quantitative.

Elemental analysis of **2**: found (%): C 52.75, H 6.05, O 8.69. Calcd. for $C_{16}H_{22}O_2TiCl_2$ (%): C 52.64, H 6.03, O 8.76.

^1H NMR (CD_2Cl_2, 298 K): 2.98 [$C_5H_4–(CH_2CH_2OCH_3)$, 2H, d]; 3.39 [$C_5H_4–(CH_2CH_2OCH_3)$, 3H, s]; 3.61 [$C_5H_4–(CH_2CH_2OCH_3)$, 2H, t]; 6.40 [$C_5H_4–(CH_2CH_2OCH_3)$, 4H, s].

^{13}C $\{^1H\}$ NMR ($CDCl_3$, 298 K): 31.41 [$C_5H_4–(CH_2CH_2OCH_3)$]; 58.68 [$C_5H_4–(CH_2CH_2OCH_3)$]; 72.16 [$C_5H_4–(CH_2CH_2OCH_3)$]; 115.99–123.34–135.41 [$C_5H_4–(CH_2CH_2OCH_3)$].

4.2.2. Synthesis of $[C_5H_4–CH_2CH_2OCH_3]_2Ti[N(CH_3)_2]_2$ (**4**)

To a solution of neutral ligand (1.42 g, 11.5 mmol) in THF (70 mL), a stoichiometic amount of potassium hydride (30 wt% dispersion in mineral oil) was slowly added at −78 °C. The solution was warmed up to room temperature and left stirred overnight, obtaining a brown potassium intermediate. Afterward the resulting solution was added dropwise to a solution of $Ti[N(CH_3)_2]_4$ (1.28 g, 5.75 mmol) in THF (10 mL) and left stirring overnight at room temperature. The KCl was removed by filtration and the resulting yellow-brown solution was concentrated to ca. 5 mL under reduced pressure. Slow addition of *n*-hexane caused the separation of a brown solid, which was washed with fresh *n*-hexane and dried in vacuum. Yield = 30%.

Elemental analysis of **4**: found (%): C 62.95, H 8.96, O 8.25 N 7.15. Calcd. for $C_{20}H_{34}N_2O_2Ti$ (%): C 62.87, H 8.90, O 8.37, N 7.33.

^1H NMR ($CDCl_3$, 298 K): 2.65 [$C_5H_4–(CH_2CH_2OCH_3)$, 2H, d]; 3.10 [$C_5H_4–(CH_2CH_2OCH_3)$, 3H, s]; 3.11 [$Ti–N(CH_3)_2$, 6H,s]; 3.41 [$C_5H_4–(CH_2CH_2OCH_3)$, 2H, t]; 5.70 [$C_5H_4–(CH_2CH_2OCH_3)$, 4H, s].

^{13}C $\{^1H\}$ NMR ($CDCl_3$, 298 K): 30.63 [$C_5H_4–(CH_2CH_2OCH_3)$]; 38.89 [$Ti–N(CH_3)_2$]; 57.75 [$C_5H_4–(CH_2CH_2OCH_3)$]; 75.78 [$C_5H_4–(CH_2CH_2OCH_3)$]; 103.7–114.7–117.3 [$C_5H_4–(CH_2CH_2OCH_3)$].

4.2.3. Synthesis of $[C_5H_4–CH_2CH_2OCH_3]_2Ti(C_2O_4)$ (**5**)

Silver oxalate (0.150 g, 0.27 mmol) and bis-methoxyethylcyclopentadienyl Ti(IV)dichloride **2** (0.100 g, 0.27 mmol) were dissolved in THF (40 mL) in a round-bottom flask, shielded from the light. The solution was left stirring for 24 h at room temperature. The suspension was gravity filtered to give a red-orange coloured filtrate. The solvent was removed in vacuum and a red-orange solid was obtained. Yield = 59.4%.

Elemental analysis of **5**: found (%): C 56.75, H 5.90, O 25.01. Calcd. for $C_{18}H_{22}O_6Ti$ (%): C 56.58, H 5.76, O 25.12.

^1H NMR (THF, D8, 298 K): 2.80 [$C_5H_4–(CH_2CH_2OCH_3)$, 2H, d]; 3.40 [$C_5H_4–(CH_2CH_2OCH_3)$, 3H, s]; 3.50 [$C_5H_4–(CH_2CH_2OCH_3)$, 2H, t]; 6.20–6.40 [$C_5H_4–(CH_2CH_2OCH_3)$, 4H,s].

^{13}C $\{^1H\}$ NMR (THF, D8, 298 K): 29.0 [$C_5H_4–(CH_2CH_2OCH_3)$]; 56.0 [$C_5H_4–(CH_2CH_2OCH_3)$]; 71.00 [$C_5H_4–(CH_2CH_2OCH_3)$]; 113–121–132 [$C_5H_4–(CH_2CH_2OCH_3)$].

4.2.4. Synthesis of $[C_5H_4–CH_2CH_2OCH_3]_2Ti(gly)_2$ (**6**)

In a round-bottom flask 60.9 mg of bis-methoxyethylcyclopentadienyl Ti(IV)dichloride **2** (16.68 mmol) was dissolved in 30 mL of

methanol (containing 1% of water). To this brown solution 0.25 mg of glycine (33.39 mmol) was added at room temperature and the mixture was stirred for 4 h. The mixture was gravity filtered and the solvent was removed from filtrate in vacuum, obtaining a yellow/brown solid. The yield was quantitative.

Elemental analysis of **6**: found (%): C 54.22, H 7.45, O 21.42, N 6.15. Calcd. for $C_{20}H_{32}O_6N_2Ti$ (%): C 54.09, H 7.20, O 21.61, N 6.30.

^{1}H NMR (MeOD, D4, 298 K): 2.80 [C_5H_4–($CH_2$$CH_2OCH_3$), 2H, d]; 3.30 [$C_5H_4$–($CH_2CH_2OCH_3$), 3H, s]; 3.57 [$C_5H_4$–($CH_2CH_2OCH_3$), 2H, t]; 6.27 [$C_5H_4$–($CH_2CH_2OCH_3$), 4H, s]; 3.60 [Ti–OCO–$CH_2$–$NH_2$, 2H, s].

^{13}C {^{1}H} NMR (MeOD, D4, 298 K): 30.49 [C_5H_4–($CH_2CH_2OCH_3$)]; 41.83 [Ti–OCO–CH_2–NH_2]; 58.0 [C_5H_4–($CH_2CH_2OCH_3$)]; 72.61 [C_5H_4–($CH_2CH_2OCH_3$)]; 115.42–116.22–117.88–118.47 [C_5H_4–($CH_2CH_2OCH_3$)].; 169.47 [Ti–OCO–CH_2–NH_2].

*4.2.5. Synthesis of [C_5H_4–$CH_2CH_2OCH_3$]$_2$$ZrCl_2$ (**7**)*

The complex was synthesized following the procedure described for complexes **2**, *i.e.* by reacting 6.5 mmol (0.800 g) of the ligand with a stoichiometric amount of *n*-BuLi (2.5 M solution in hexane, 2.6 mL) in THF and adding the resulting lithium derivative to 3.25 mmol (0.757 g) of $ZrCl_4$ dissolved in 20 mL of THF at −78 °C. The reaction mixture was left under stirring overnight at room temperature, then refluxed for 3 h and then it was filtered to remove LiCl. The solvent was evaporated at reduced pressure and the grey solid dried in vacuum. Yield = 68%.

Elemental analysis of **7**: found (%): C 47.22, H 5.52, O 7.56. Calcd. for $C_{16}H_{22}O_2ZrCl_2$ (%): C 47.06, H 5.39, O 7.83.

^{1}H NMR ($CDCl_3$, 298 K): 2.89 [C_5H_4–($CH_2CH_2OCH_3$), 2H, d]; 3.32 [C_5H_4–($CH_2CH_2OCH_3$), 3H, s]; 3.58 [C_5H_4–($CH_2CH_2OCH_3$), 2H, t]; 6.27–6.29 [C_5H_4–($CH_2CH_2OCH_3$), 4H, s].

^{13}C {^{1}H} NMR ($CDCl_3$, 298 K): 31.02 [C_5H_4–($CH_2CH_2OCH_3$)]; 58.84 [C_5H_4–($CH_2CH_2OCH_3$)]; 72.87 [C_5H_4–($CH_2CH_2OCH_3$)]; 113.1–118.0–132.2 [C_5H_4–($CH_2CH_2OCH_3$)].

4.3. Hydrolysis tests

NMR samples were prepared by transferring 5 mg of appropriate compound under a N_2 flush into a 10-mm NMR tube that had been charged with 1.00 mL of DMSO (or solution DMSO/D_2O: 9/1) previously saturated with N_2 for several minutes. ^{1}H NMR spectra were then recorded at various time intervals with the sample maintained at 37 °C.

4.4. Pharmacology

4.4.1. Cell lines and culture conditions

Human breast cancer (MCF-7), human embryonic kidney (HEK-293) and murine macrophage (J774.A1) cell lines were maintained and grown in adhesion on Petri dishes with DMEM supplemented with FCS (10%), hepes (25 mM), penicillin (100 u/mL) and streptomycin (100 units/mL).

4.4.2. MTT assay for antiproliferative activity

MCF-7, HEK-293 and J774.A1 (3.5×10^4 cells/well) were plated on 96-well microtiter plates and allowed to adhere at 37 °C in a 5% CO_2 atmosphere for 2 h. Thereafter, the medium was replaced with fresh one (50 μL) and a 75 μL of 1:4 serial dilution of each tested compound was added, and then the cells incubated for further 72 h. *cis*-Platinum was used as reference drug. Mitochondrial respiration, an indicator of cell viability, was assessed by the mitochondrial-dependent reduction of [3-(4,5-dimethylthiazol-2-yl)-2,5-phenyl-2H-tetrazolium bromide] (MTT) to formazan and cells viability was assessed accordingly to the method of Mosmann [42]. Briefly 25 μL of MTT (5 mg/mL) was added and the cells were incubated for an additional 3 h. Thereafter, cells were lysed and the dark blue crystals solubilised with 100 μL of a solution containing 50% (v:v) N,N-dimethylformamide, 20% (w:v) SDS with an adjusted pH of 4.5. The optical density (OD) of each well was measured with a microplate spectrophotometer (Titertek Multiskan MCC/340) equipped with a 620 nm filter. The viability of each cell line in response to treatment with tested compounds and 6-mercaptopurine was calculated as: % dead cells = 100–(OD treated/OD control) × 100.

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