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Study of the anticancer properties of methyl- and phenyl-substituted carbon- and silicon-bridged *ansa*-titanocene complexes

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ABSTRACT

The previously known complexes [Ti{(Me_2CMe_2C)(η^5-C_5H_4)$_2$}Cl_2] (**1**), [Ti{Me_2C(η^5-C_5H_4)$_2$}Cl_2] (**2**), [Ti{Me_2Si(η^5-C_5H_4)$_2$}Cl_2] (**4**), [Ti{MePhSi(η^5-C_5H_4)$_2$}Cl_2] (**5**) and [Ti{MePhSi(η^5-C_5Me_4)$_2$}Cl_2] (**6**) have been prepared following reported procedures. The novel complex [Ti{MePhC(η^5-C_5H_4)$_2$}Cl_2] (**3**) has been prepared and characterized. The cytotoxic activity of **1**–**6** has been tested after 72 h on melanoma A375 and B16, prostate cancer DU145 and LNCaP and colon cancer HCT116, SW620 and CT26CL25 cell lines observing a high cytotoxic activity of complexes **1** and **6** compared to the reference compound ([Ti(η^5-C_5H_5)$_2$}Cl_2]). **1** and **6** have also been tested against primary normal mouse keratinocytes and lung fibroblasts. While viability of both type of primary cells was significantly less affected by **1** in comparison to the reference compound [Ti(η^5-C_5H_5)$_2$$Cl_2$], compound **6** was completely nontoxic for nonmalignant cells, indicating a potential selectivity of this compound towards cancer cell lines. In addition CFSE staining, cell cycle analysis, AnnexinV-FITC/PI staining, detection of caspase activity and mitochondrial potential showed that **1** and **6** were acting through inhibition of proliferation and subsequent induction of mitochondrial dependent apoptosis in colon cancer cell lines, HCT116 and SW620, which express low sensitivity to cisplatin. Compound **6** was found to be the leading drug in this group since it shows the fastest and most selective anticancer profile.

1. Introduction

Titanocene derivatives are together with ferrocene complexes the most studied metallocenes in preclinical trials in the treatment of cancer [1]. The starting point of this research field was the work of Köpf and Köpf-Maier which showed the cytotoxic properties of titanocene dichloride, [Ti(η^5-C_5H_5)$_2$$Cl_2$] and some of its analogs in the early 1980's [2]. Titanocene dichloride was studied in phase I clinical trials in 1993 [3–5] and some water soluble formulations were subsequently developed by Medac GmbH (Germany) [6]. Phase I clinical trials were not as satisfactory as expected, but the research in the topic continued with some phase II clinical trials in patients with breast metastatic carcinoma [7] and advanced renal cell carcinoma [8], which showed a low activity discouraging further studies.

However, after the recent work of many different groups worldwide, the interest in this topic has been renewed [9–13]. Thus, aminoacid-functionalized titanocene derivatives [14,15], benzyl-substituted titanocene or *ansa*-titanocene complexes [13], ionic titanocene compounds [16,17], alkylammonium-substituted titanocene derivatives [18–20], steroid-functionalized titanocenes [21], and alkyl-, aryl- and/or alkenyl-substituted titanocene or *ansa*-titanocene derivatives [22–25] have been synthesized, characterized and studied in preclinical trials against different types of human cancer cell lines and primary cells.

In general, the cytotoxic activity of titanocene complexes has been correlated to their structure, and the study of the anticancer mechanism of titanocene derivatives is currently a very active research field. Several studies have led to the proposal that after hydrolysis of titanocene complexes, titanium ions reach the cell

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cytoplasm assisted by the major iron transport protein "transferrin" [26–29]. Inside the cell, titanium ions are transported to the nucleus (probably assisted by ATP) and then bind to DNA and lead to cell death [30–32]. However, recent experiments have also reported on the potential interaction of a ligand-bound Ti(IV) complex to other proteins [33–35], which may also be implicated in the induction of cell death.

There are several reports on the cytotoxicity of titanocene derivatives with different substituents attached to Cp rings [1,9,13], but not many studies on the mechanistic properties of alkyl- or alkenyl-substituted silicon- or carbon-bridged *ansa*-titanocene derivatives [22,23]. With the aim to contribute to the understanding of the mechanism of cancer cell death promoted by silicon- or carbon-bridged *ansa*-titanocene derivatives with simple alkyl or aryl substituents, a complete study which consists of the synthesis, characterization, cell cycle analysis, AnnexinV-FITC/PI staining and caspase detection has been carried out and is reported here.

2. Experimental section

2.1. General manipulations

All reactions were performed using standard Schlenk tube techniques in an atmosphere of dry argon. Solvents were distilled from the appropriate drying agents and degassed before use. [Ti{(Me_2CMe_2C)(η^5-C_5H_4)$_2$}Cl_2] (**1**) [36], [Ti{Me_2C(η^5-C_5H_4)$_2$}Cl_2] (**2**) [37], [Ti{Me_2Si(η^5-C_5H_4)$_2$}Cl_2] (**4**) [38], [Ti{MePhSi(η^5-C_5H_4)$_2$}Cl_2] (**5**) [39] and [Ti{MePhSi(η^5-C_5Me_4)$_2$}Cl_2] (**6**) [39] were prepared according to previously described synthetic methods. Reference compound titanocene dichloride ([Ti(η^5-C_5H_5)$_2$}Cl_2] (**Ref**)) was purchased from Sigma–Aldrich (Spain).

Acetophenone, NaOH, NH_4Cl, Na_2SO_4, LiBu^n (solution 2.5 M in hexanes) and $TiCl_4$ were purchased from Aldrich. All the commercial reagents were used directly without further purification. IR spectra were recorded on a Thermo Nicolet Avatar 330 FT-IR spectrophotometer. ^{1}H and ^{13}C{^{1}H} NMR spectra were recorded on a Varian Mercury 300 spectrometer in $CDCl_3$ solutions at 298 K. Chemical shifts (δ/ppm) are given relative to solvent signal (δ_H: 7.26 ppm; δ_C: 77.16 ppm). 2D NMR experiments such as ^{1}H–^{1}H COSY and ^{13}C–^{1}H HSQC have been carried out for the correct assignation of the signals. Microanalyses were carried out with a Perkin–Elmer 2400 or LECO CHNS-932 microanalyzer. Mass spectroscopic analyses were performed on a VG7070E spectrometer at 70 eV.

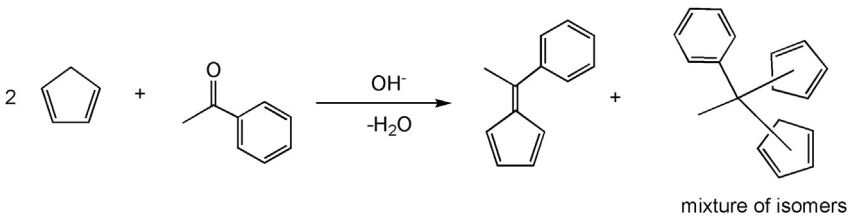
2.2. Synthesis of [Ti{MePhC(η^5-C_5H_4)$_2$}Cl_2] (**3**)

The first step for the preparation of compound **3** is the synthesis of MePhC(C_5H_5)$_2$ according to Scheme 1.

The ligand was prepared from cyclopentadiene and acetophenone in the presence of NaOH as a base, according to a reported method [40].

The freshly cracked cyclopentadiene (93.0 g, 1.4 mol) was added to a suspension of NaOH (112.0 g, 2.8 mol) in 400 mL of THF. The mixture was stirred for 1 h and then acetophenone (84.0 g, 0.7 mol) was added dropwise during 30 min. The resulting red–orange mixture was stirred for 14 h and then refluxed for 5 h. The dark red–orange solution was separated and residual NaOH suspension was washed twice with 120 mL of diethyl ether. Collected organic phases were combined and the resulting solution was washed subsequently with an NH_4Cl solution, brine and finally with water to neutral reaction and dried over Na_2SO_4. The solution was filtered, volatiles were evaporated on rotary evaporator and the red–orange oily residue was fractionally distilled at reduced pressure (0.01 mm Hg). The first fraction up to 80 °C contained almost exclusively 6-methyl-6-phenylfulvene as was determined by GC–MS and ^{1}H NMR spectrometry. The fraction collected at 118–125 °C contained mainly the desired product in ca 90% purity besides ca. 10% of 6-methyl-6-phenylfulvene as was determined by GC–MS. The residual 6-methyl-6-phenylfulvene was distilled off from the mixture at reduced pressure (0.01 mm Hg) using a boiling water bath, leaving product as an orange oily liquid. Yield 39.1 g (24%). For this synthesis, the temperature of the heating bath should be kept below 130 °C during distillation, otherwise the polymerization of the crude product could be initiated. Elemental analysis $C_{18}H_{18}$, calculated C, 92.26; H, 7.74%, found C, 92.35; H 7.80%. GC–MS, *m*/*z* (relative abundance): 235 (19), 234 ($M^{+\bullet}$; 100), 219 ([M – Me]$^+$; 77), 205 (23), 204 (45), 203 (36), 202 (25), 191 (35), 178 (33), 169 (25), 168 (21), 165 (22), 154 (24), 153 (30), 152 (26), 142 (19), 141 (30), 128 (21), 115 (30), 91 (25), 77 (11). After the preparation of MePhC(C_5H_5)$_2$ the synthesis of [Ti{MePhC(η^5-C_5H_4)$_2$}Cl_2] (**3**) was carried out according to Scheme 2.

LiBu^n (28.8 mL, 2.5 M, 72 mmol) was dropped to a solution of the ligand MePhC(C_5H_5)$_2$ (8.42 g, 36 mmol) in THF (100 mL). The resulting red–orange mixture was stirred for 3 h and then slowly transferred to another Schlenk vessel containing cold (−75 °C) suspension of $TiCl_4(THF)_2$ (prepared *in situ* by reaction of $TiCl_4$ (4.0 mL, 36 mmol) with 6 mL of THF) in 100 mL of toluene. The mixture was allowed to warm to room temperature and then heated to 65 °C for 16 h. The resulting dark red mixture was evaporated almost to dryness and treated with 200 mL of hexane. The formed brown precipitate was isolated, washed with hexane (3 × 60 mL) and dried on air. The solid was extracted in boiling dichloromethane in a Soxhlet extractor and then recrystallized from hexane. The title complex was obtained as a dark brown crystalline solid. Yield: 0.8 g (6%). Elemental analysis $C_{18}H_{16}Cl_2Ti$, calculated C, 61.58; H, 4.59%, found C, 61.39; H 4.51%. M.p. 168 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 1.99 (s, 3H, C*Me*); 5.52–5.57 (m, 2H, C(5)*H*, C_5H_4); 5.74–5.78 (m, 2H, C(2)*H*, C_5H_4); 6.98–7.05 (m, 4H, C(3)*H* and C(4)*H*, C_5H_4); 7.38 (tt, $^3J_{HH}$ = 7.5 Hz, $^4J_{HH}$ = 1.2 Hz, 1H, CH_{para}, *Ph*); 7.44–7.52 (m, 2H, CH_{meta}, *Ph*); 7.61–7.67 (m, 2H, CH_{ortho}, *Ph*). ^{13}C{^{1}H}($CDCl_3$): 27.24 (C*Me*); 48.49 (*C*Me); 112.94 (*C*(2), C_5H_4); 113.14 (*C*(5), C_5H_4); 114.98 (*C*(1), C_5H_4); 128.07 (*C*H$_{ortho}$, *Ph*); 128.11 (*C*H$_{para}$, *Ph*); 129.56 (*C*H$_{meta}$, *Ph*); 129.73 (*C*(3), C_5H_4); 133.48 (*C*(4), C_5H_4). EI-MS, *m*/*z* (relative abundance): 354 (16), 353 (19), 352 (73), 351 (32), 350 ($M^{+\bullet}$, 100), 316 (40), 315 (46), 314 ([M – HCl]$^+$, 88), 313 (18), 299 ([M – HCl – Me]$^+$, 12), 298 (10), 280 (12), 279 (23), 278 ([M – 2HCl]$^+$, 83), 277 (18), 276 (20), 263 ([M – 2HCl – Me]$^+$, 15), 216 (11), 215 (30), 165 (18), 153 (15), 152 (22), 147 (30), 139 (20),



Scheme 1. Synthesis of the carbon-bridged bis-cyclopentadienyl ligand.

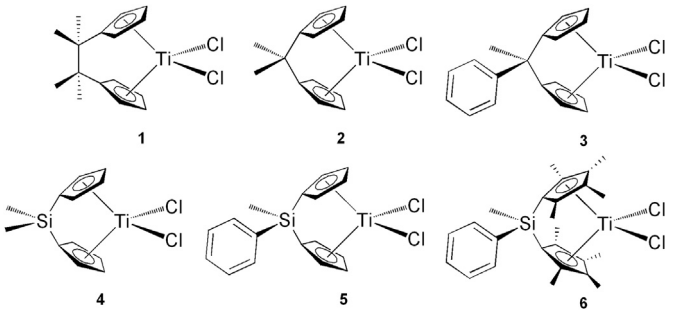


Fig. 1. Titanocene complexes used in this study.

115 (18). IR (KBr): 3103 (w); 3088 (m); 3056 (vw); 3027 (vw); 2976 (m); 2933 (m); 2873 (vw); 1558 (vw); 1541 (vw); 1494 (m); 1474 (w); 1464 (w); 1448 (m); 1417 (m); 1378 (w); 1276 (m); 1243 (w); 1105 (m); 1075 (w); 1040 (w); 1025 (m); 918 (w); 815 (vs); 767 (s); 727 (s); 703 (vs); 633 (vw); 594 (vw); 574 (m); 463 (w); 427 (w).

2.3. Reagents, cells and animals

Fetal calf serum (FCS), RPMI-1640, phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), collagenase IV from *Clostridium Hystoliticum*, and propidium iodide (PI) were obtained from Sigma (St. Louis, MO). AnnexinV-FITC (AnnV) was from Biotium (Hayward, CA). Acridin orange (AO) was from Labo-Moderna (Paris, France). The B16 murine melanoma was kind provided by Dr. Sinisa Radulovic (Institute for Oncology and Radiology of Serbia, Belgrade, Serbia) while human melanoma A375, colon cancer HCT116 and SW620, prostate LNCaP and DU145 and mouse colon CT26CL25 were kindly provided by Prof. Ferdinando Nicoletti (Department of Biomedical Sciences, University of Catania, Italy).

Cells are routinely maintained in HEPES-buffered RPMI-1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 0.01% sodium pyruvate, and antibiotics (culture medium) at 37 °C in a humidified atmosphere with 5% CO_2. After standard trypsinization, cells were seeded at 5×10^3/well in 96-well plates for viability determination and 2.5×10^5/well in 6-well plate for flow cytometry.

For isolation of fibroblasts and keratinocytes, 2.5 month old C57BL6 female mices were used. Animals were bred and kept in our own facility of the Institute for Biological Research "Sinisa Stankovic" (Belgrade, Serbia) under standard laboratory conditions (nonspecific pathogen free) with free access to food and water. All procedures were approved by the local Institutional Animal Care and Use Committee.

2.4. Preparation of keratinocytes

Mouse ears were split into two pieces and exposed to 0.5% trypsin-PBS solution for 1 h at 37 °C then epidermis was taken and cut into small pieces. Cells were resuspended and filtered through nylon meshes. Cells were finally resuspended into RPMI-15% FCS.

2.5. Preparation of adult mouse lung fibroblasts

Lungs were aseptically removed, rinsed in PBS and cut into small pieces. Tissue was then digested in collagenase IV from *C. Hystoliticum* (0.7 mg/mL) + DNase (30 mg/mL) with slow stirring for 1 h at 37 °C. Cells were washed three times and resuspended in RPMI-15% FCS. After 24 h non-adherent cell were discarded and medium was replaced. Cells that reached 80–90% of confluence were used for experiments.

2.6. Determination of cell viability by MTT and crystal violet assay

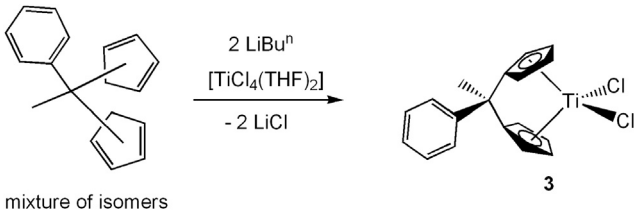
The viability of adherent viable cells was evaluated with crystal violet (CV) assay, while mitochondrial dehydrogenase activity, as indicator of cell viability, was used for non-adherent cells. Cells (5×10^3/well) were treated with the wide range of doses of the drugs for 72 h and viability was measured as described [41]. In brief, at the end of cultivation period adherent cells were fixed with methanol 10 min at RT and subsequently stained 15 min with 1% CV solution. Then, the cells were washed, dried and the dye was dissolved in 33% acetic acid. The absorbance of dissolved dye was measured at 540 nm with the reference wavelength at 640 nm. For determination of viability of non-adherent cells, MTT was added to cell cultures in a final concentration of 0.5 mg/mL. Cells were collected after 2 h, centrifuged and pellets were dissolved in DMSO. The absorbance that corresponds to the level of MTT reduction to formazan, was measured at 540 nm with the reference wavelength at 640 nm. Results are calculated as percentage of control that was arbitrarily set to 100%. All tests were carried out in triplicate cultures and repeated three times. IC_{50} value was calculated using the following formula:

$$IC_{50} = \frac{(50\% - \text{Low Inh}\%)}{(\text{High Inh}\% - \text{Low Inh}\%) \times (\text{High Conc} - \text{Low Conc})} + \text{Low Conc}$$

where Low Inh%/High Inh% is % inhibition directly below/above 50% inhibition and Low Conc/High Conc is the corresponding concentrations of test compound, presented as mean ± SD from three independent experiments.

2.7. Cell cycle analysis

Cells (2.5×10^5/well) were treated with IC_{50} dose (87 and 98 μM of **1**, 75 and 68 μM of **6** and 142 and 200 μM of **Ref** for SW620 and HCT116 cells, respectively) for 24 h, then trypsinized and fixed in 70% ethanol at 4 °C for 30 min. After washing in PBS, cells were incubated with PI (20 μg/mL) and RNase (0.1 mg/mL) for 30 min at 37 °C in dark. Red fluorescence was analyzed with FACS Calibur



Scheme 2. Synthesis of titanocene complex **3**.

Table 1
IC_{50} [μM] values of titanium(IV) complexes **1**–**6** and reference complex, **Ref** = [Ti(η^5-C_5H_5)$_2$Cl$_2$] (72 h of action against selected tumor cell lines). IC_{50} values were calculated as mean ± SD from three independent experiments.

Cell line/Compound	**Ref**	**1**	**2**	**3**	**4**	**5**	**6**
A375	161 ± 1	124 ± 36	152 ± 7	127 ± 15	170 ± 17	181 ± 9	105 ± 29
B16	>200	86 ± 7	182 ± 1	178 ± 19	>200	>200	43 ± 4
HCT116	>200	98 ± 19	148 ± 1	144 ± 25	>200	160 ± 1	68 ± 6
SW620	141 ± 1	87 ± 2	>200	132 ± 18	158 ± 1	199 ± 2	75 ± 1
CT26CL25	154 ± 1	119 ± 3	>200	148 ± 24	163 ± 1	>200	62 ± 13
DU145	163 ± 36	93 ± 34	175 ± 1	117 ± 43	142 ± 1	156 ± 1	83 ± 24
LnCap	>200	100 ± 20	>200	163 ± 11	197 ± 1	>200	66 ± 19

flow cytometer (BD, Heildelberg, Germany). The distribution of cells in different cell cycle phases was determined with Cell Quest Pro software (BD).

2.8. AnnexinV-FITC/PI staining and caspase detection

Cells (2.5 × 10^5/well) were treated with IC_{50} dose of each compound (87 and 98 μM of **1**, 75 and 68 μM of **6** and 142 and 200 μM of **Ref** for SW620 and HCT116 cells, respectively) for 48 or 72 h, then trypsinized and stained with AnnV-FITC/PI (Biotium, Hayward, CA) or apostat (R&D Systems, Minneapolis, MN USA) according to the manufacturer's instructions. Cells were analyzed with FACS Calibur flow cytometer (BD, Heidelberg, Germany) using Cell Quest Pro software (BD).

2.9. CFSE staining

Cells were stained with 1 μM of carboxyfluorescein succinimidyl ester (CFSE) for 10 min at 37 °C, then washed and treated with IC_{50} dose of each compound (87 and 98 μM of **1**, 75 and 68 μM of **6** and 142 and 200 μM of **Ref** for SW620 and HCT116 cells, respectively) for 72 h. At the end of cultivation cells were trypsinazed, washed and analyzed with FACS Calibur flow cytometer.

2.10. JC-1 staining

Cells (2.5 × 10^5/well) were treated with IC_{50} dose of each compound (87 and 98 μM of **1**, 75 and 68 μM of **6** and 142 and 200 μM of **Ref** for SW620 and HCT116 cells, respectively) for 72 h, then trypsinized and stained with JC-1 dye (5 μg/mL) (Biotium) for 20 min at 37 °C. Finally, cells were washed, resuspended in PBS and analyzed with FACS Calibur flow cytometer.

2.11. Statistical analysis

The results of viability assays are presented as mean ± SD of triplicate observations from one representative of at least three experiments with similar results. The significance of the differences between various treatments was determined by ANOVA followed by Student Newman–Keuls test. $P < 0.05$ was considered to be significant.

3. Results and discussion

3.1. Synthesis and characterization

[Ti{(Me_2CMe_2C)(η^5-C_5H_4)$_2$}Cl$_2$] (**1**) [36], [Ti{Me_2C(η^5-C_5H_4)$_2$}Cl$_2$] (**2**) [37], [Ti{Me_2Si(η^5-C_5H_4)$_2$}Cl$_2$] (**4**) [38], [Ti{MePhSi(η^5-C_5H_4)$_2$}Cl$_2$] (**5**) [39] and [Ti{MePhSi(η^5-C_5Me_4)$_2$}Cl$_2$] (**6**) [39] (Fig. 1) were prepared according to previously described synthetic methods.

For the preparation of the carbon-bridged *ansa*-titanocene [Ti{MePhC(η^5-C_5H_4)$_2$}Cl$_2$] (**3**), the synthesis of the corresponding ligand MePhC(C_5H_5)$_2$ was carried out according to literature procedures (Scheme 1). This ligand was di-lithiated by treatment with two equivalents of $LiBu^n$ and subsequently reacted with one equivalent of [$TiCl_4$(THF)$_2$] (Scheme 2).

The NMR, mass and IR spectra and elemental analysis showed that all the complexes (**1**–**6**), isolated as crystalline solids, which were of high purity.

For the new complex [Ti{MePhC(η^5-C_5H_4)$_2$}Cl$_2$] (**3**) the ^{1}H NMR spectrum shows three multiplets between 5.52 and 7.05 ppm with 1:1:2 intensity ratio which correspond to the four magnetically unequivalent protons of the cyclopentadienyl rings. In addition, a singlet at 1.99 ppm (corresponding to the protons of the methyl group of the bridge) and three multiplets between 7.38 and 7.67 ppm (assigned to the protons of the phenyl group) were also observed.

The $^{13}C\{^1H\}$ NMR spectrum shows the expected signals for the different carbon atoms of the complex (see Experimental section 2.2). Characterization of **3** by mass spectrometry shows the molecular ion peak as the base peak of the spectrum.

3.2. Biological studies

3.2.1. Effect of titanium complexes on tumor cell viability

Antitumor potential of titanium complexes (**1**–**6**) and the reference compound [Ti(η^5-C_5H_5)$_2$Cl$_2$] (**Ref**) on melanoma A375 and B16, prostate cancer DU145 and LNCaP and colon cancer SW620, HCT116 and CT26CL25 cell lines was evaluated by the treatment with [Ti(η^5-C_5H_5)$_2$Cl$_2$] and its analogs (**1**–**6**). Cell viability was determined by CV after 72 h of treatment.

The obtained results (Table 1) clearly indicate that two of the studied compounds, namely **1** and **6**, showed improved antitumor potential in comparison to the reference compound titanocene dichloride (**Ref**) except in the case of A375 cells.

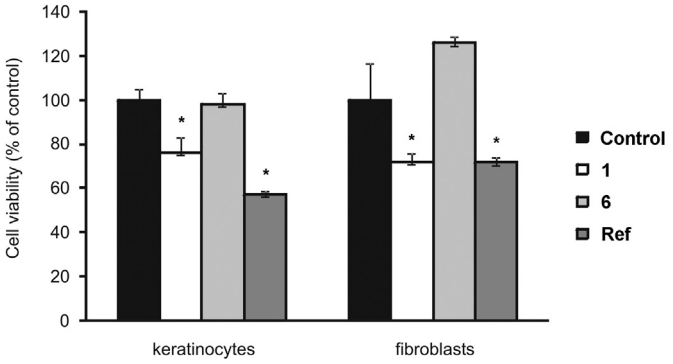


Fig. 2. The effect of titanocene complexes on viability of normal cells. (A) Keratinocytes (8 × 10^4 cells/well) and (B) fibroblasts (3 × 10^4 cells/well) were treated with 90 and 70 μM of 1 and 6 and 200 μM of **Ref** for 72 h, after which cell viability was determined by MTT and CV assay, respectively. The data are presented as mean ± SD from representative of three independent experiments.

From these results one can see that incorporation of an ethylene *ansa*-bridge increases the cytotoxicity as it is confirmed by the much lower IC_{50} values associated to **1** compared to its analog **2** (with just a single carbon atom in the *ansa*-bridge).

In addition, incorporation of a phenyl ring attached directly to the bridging atom of the carbon-bridged titanocene derivatives, **3**, increases the cytotoxicity, indicated by the IC_{50} values which are lower than those of **2** in all the studied cancer cell lines. However, this behavior seems to induce opposite effect regarding *in vitro* activity in the silicon-bridged compounds, because **4** demonstrated lower IC_{50} values than titanocene compound **5** (except in the HTC116 cell line).

Finally, as reported in our recent work [23], the incorporation of tetramethyl-substituted silicon-bridged *ansa*-biscyclopentadienyl ligands as in the case of complex **6**, increases the cytotoxic activity in comparison with their non-substituted analogs (**4** and **5**).

Complexes **2**–**5** are less active compared to **1** and **6** due to the non-substitution of cyclopentadienyl rings, as it was previously observed by our group [22,23]. This substitution decreases the hydrophobic nature of the complexes, and thus the possibility of formation of micellar species decreasing the stability of the Ti-cyclopentadienyl bonds, leading to a higher degree of hydrolysis which may induce a lower cytotoxic activity. This phenomenon is counterbalanced in complex **1** due to the incorporation of an ethylene *ansa*-bridge which increases the cytotoxicity.

The highest activity of complexes **1** and **6** relative to the reference compound (**Ref**) has been observed against B16, HCT116, SW620 and CT26CL25 cancer cells. Importantly, the compounds were efficient against cell lines low responsive to cisplatin such as HCT116 and SW620. These results indicate that treatment with **1** and **6** may overcome the problems associated to the development of resistance of the cancer cells to cisplatin.

Compounds **1** and **6** have higher cytotoxicity than titanocene dichloride but only modest cytotoxicity in comparison to the results reported for highly active titanocene compounds like titanocene-Y or some other analogs [13]. However, we decided to study some mechanistic aspects of these compounds which were subsequently analyzed.

3.2.2. *Effect of complexes **1** and **6** on primary cells*

In view of the interesting results obtained with complexes **1** and **6**, an evaluation of the selectivity of these compounds by the study of their action against primary mouse keratinocytes and lung fibroblasts was carried out. Primary cells were treated with 90 and 70 μM solutions of **1** and **6**, respectively. The chosen doses are average IC_{50} calculated from the values obtained on relevant cell lines (B16, HCT116 and SW620). Cell viability was determined by CV (for fibroblasts) and MTT assays (for keratinocytes) after 72 h of treatment. In the same time frame, viability of keratinocytes was significantly less affected by **1** than by the reference compound $[Ti(\eta^5\text{-}C_5H_5)_2Cl_2]$ (**Ref**). On the other hand, IC_{50} concentration of **6** was completely nontoxic to both keratinocytes and fibroblasts, indicating a potential selectivity of this compound towards cancer cell lines (See Fig. 2).

3.2.3. *Antitumor action mechanism of **1** and **6***

HCT116 and SW620 cells (IC_{50} for cisplatin >120 μM) were selected for further analysis To determine the cause of decreased viability, colon cancer HCT116 and SW620 cell lines were treated with IC_{50} doses of **1** and **6** and the reference compound $[Ti(\eta^5\text{-}C_5H_5)_2Cl_2]$ (**Ref**) determined for each cell line, and after 72 h cell cycle distribution was analyzed.

As presented on Fig. 3A and B (left panel) treatment with **1** and **6** resulted in accumulation of hypodiploid cells in subG compartment in both cell lines indicated proapoptotic pattern of their anticancer activity. The effect of **6** on SW620 cells was the most pronounced in comparison to all the other studied compounds, while reference compound $[Ti(\eta^5\text{-}C_5H_5)_2Cl_2]$ (**Ref**) promoted DNA fragmentation

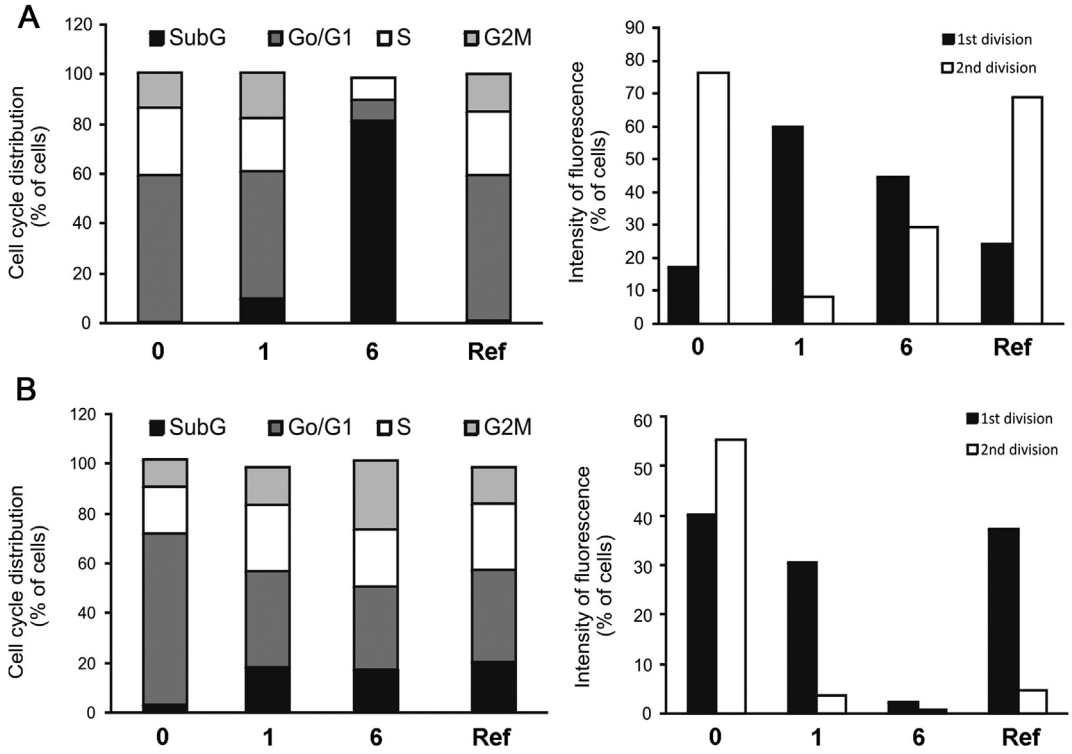


Fig. 3. The effect of titanocene complexes on cell cycle progression. (A) SW620 cells and (B) HCT116 cells (2×10^5/well) were exposed to IC_{50} doses of each compound (87 and 98 μM of **1**, 75 and 68 μM of **6** and 142 and 200 μM of **Ref** for SW620 and HCT116 cells, respectively) for 72 h and cell cycle distribution (left panel) was evaluated. For determination of cellular proliferation (right panel) cells were pre-stained with CFSE and then exposed to treatment as indicated above.

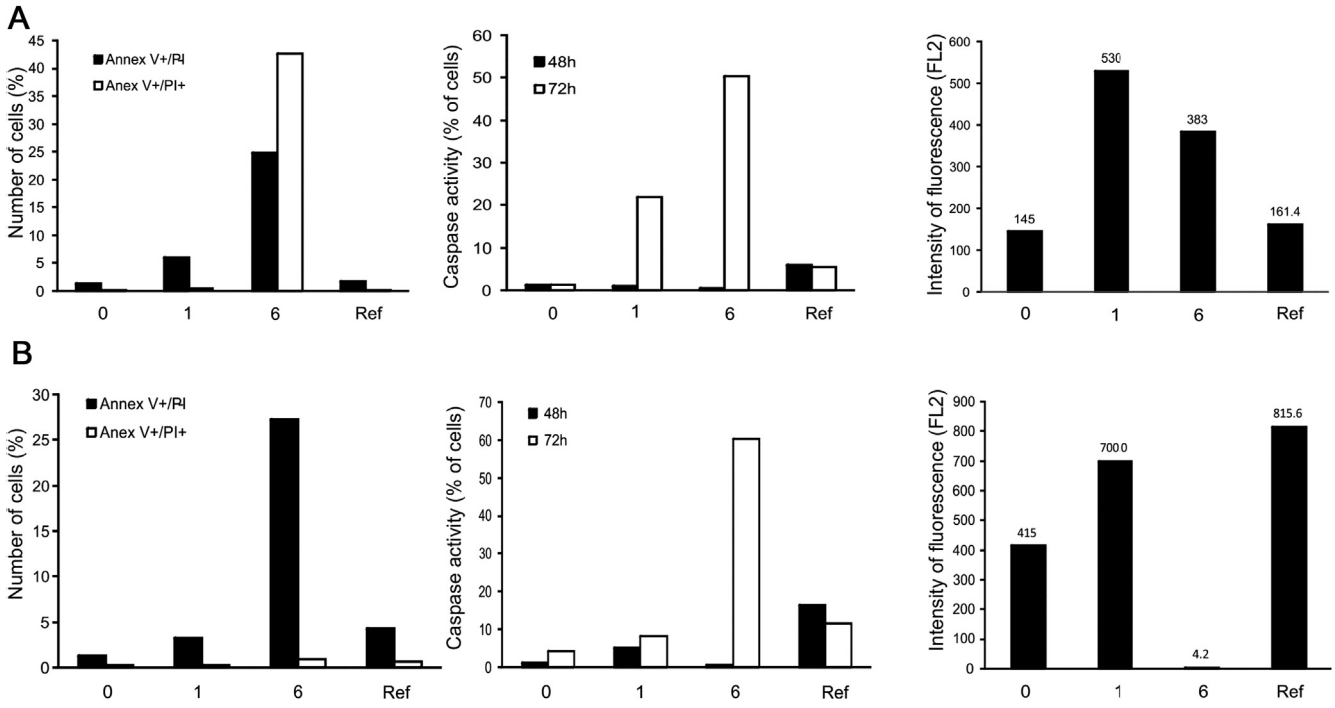


Fig. 4. The effect of Ti complexes on apoptotic process. (A) SW620 cells and (B) HCT116 cells (2×10^5/well) were exposed to IC_{50} doses of each compound (87 and 98 μM of compound **1**, 75 and 68 μM for compound **6** for SW620 and HCT116 cells, 142 and 200 μM **Ref** respectively) and Ann/PI double staining after 48 h, ApoStat staining after 48 and 72 h and mitochondrial depolarization after 72 h were determined.

only in HCT116 cells. At the same time point, analysis of cellular proliferation by CFSE staining, revealed significant inhibition of cell division although the kinetic was different in the studied cell lines. While on SW620 cells proliferation upon the treatment with both compounds was blocked after the second division, number of divided HCT116 cells significantly decreased already in the first division. These results indicated that titanocene derivatives worked through inhibition of proliferation and further induction of apoptosis (Fig. 3A and B, right panel).

To confirm apoptosis as major mechanism responsible for decreased viability of SW620 and HCT116 cells, Ann/PI double staining was performed after 48 h of incubation with tested compounds. Obtained results showed intensive accumulation of early apoptotic cells in HCT116 and SW620 cells exposed to both agents. The effect of **6** was dominant and faster in comparison to **1** as judged by the elevated presence of double positive late apoptotic cells (Fig. 4). On the other hand, negative results of LDH release assay for determination of necrotic cell death in the first 24 h of incubation (not shown), eliminates the possibility of necrosis being the pivotal mode of drug action. The apoptotic process confirmed by cell cycle analysis as well as Ann/PI staining was accompanied with caspase activation. This phenomenon was time-dependent and dominant in cultures exposed to **6**. To precise the intracellular pathway involved in the induction of the apoptotic process, mitochondrial membrane potential was estimated by JC-1 staining. A 72 h long treatment with **1**, triggered hyperpolarization of mitochondrial membrane in both cell lines. However, same experimental conditions using **6** led to mitochondrial membrane collapse in HCT116. These results showed again a faster and more potent activity of **6** relative to compounds **1** and **Ref**.

It is well known that apoptotic process is most commonly associated with the collapse of mitochondrial membrane potential. However, hyperpolarization often precedes the total mitochondrial collapse and therefore can be considered as an early sign of increased apoptosis [42]. Apoptosis is often a trigger for an autophagic process, salvaging pathway in response to stress. However, depending on the conditions, this process managed cell death. Unchanged presence of autophagosomes, determined by AO supravital staining, indicated that autophagic process is irrelevant for drug action, even in cytoprotective or destructive manner (not shown).

4. Conclusions

The novel complex [Ti{MePhC(η^5-C_5H_4)$_2$}Cl_2] (**3**) has been synthesized and characterized by traditional methods. The cytotoxicity of **3** and the known complexes [Ti{(Me_2CMe_2C)(η^5-C_5H_4)$_2$}Cl_2] (**1**), [Ti{Me_2C(η^5-C_5H_4)$_2$}Cl_2] (**2**), [Ti{Me_2Si(η^5-C_5H_4)$_2$}Cl_2] (**4**), [Ti{MePhSi(η^5-C_5H_4)$_2$}Cl_2] (**5**) and [Ti{MePhSi(η^5-C_5Me_4)$_2$}Cl_2] (**6**) and the reference compound [Ti(η^5-C_5H_5)$_2Cl_2$] (**Ref**) has been tested against seven different cancer cell lines.

An increase in the cytotoxic activity has been observed by the incorporation of an ethylene *ansa*-bridge in the titanocene derivative (complex **1**), compared to its analogous with just a single carbon atom in the *ansa*-bridge (complex **2**). In addition, it seems that the incorporation of a phenyl ring attached directly to the bridging atom decreases the viability of the cancer cells in carbon-bridged compounds, while increases the viability of the cancer cells in silicon-bridged systems. Furthermore, one can envisage that the incorporation of tetramethyl-substituted silicon-bridged *ansa*-biscyclopentadienyl ligands significantly decreases the viability of the cancer cells, in comparison with their non-substituted silicon-bridged *ansa*-biscyclopentadienyl analogs. However, all these proposed structure–activity relationships are subjected to further tests that should be carried out using more complexes of this type.

Complexes **1** and **6** are the most cytotoxic of all the analyzed complexes against all the studied cancer cell lines with IC_{50} values of up to 43 ± 4 μM (for **6** against B16 cancer cell). All the studied compounds are generally more cytotoxic than the reference compound [Ti(η^5-C_5H_5)$_2Cl_2$] (**Ref**). Of particular importance is activity of complexes **1** and **6** against cisplatin non-sensitive clones of colon

cancer cells HCT116 and SW620, indicating that these compounds may overcome the problems associated to the development of resistance of the cancer cells to cisplatin.

The most cytotoxic compounds, **1** and **6** have also been tested against primary mouse keratinocytes and lung fibroblasts observing that viability of both types of primary cells were significantly less affected by **1** than by the reference compound titanocene dichloride. In addition, **6** was completely nontoxic to primary cells, indicating that possesses a potential selectivity.

Finally, the mechanism of action of **1** and **6** on cisplatin resistant colon cancer HCT116 and SW620 cell lines has been tested by using cell cycle analyses. The results show that treatment with **1** and **6** resulted in accumulation of hypodiploid cells in subG compartment in both cell lines, carrying out their action through inhibition of proliferation and further induction of apoptosis. Treatment with **1** triggered hyperpolarization of mitochondrial membrane in both cell lines, while **6** led to mitochondrial membrane collapse in HCT116.

While SW620 cell proliferation when treating them with **1** and **6** showed a blocking after the second division, number of divided HCT116 treated cells significantly decreased already in the first division. These results showed that titanocene derivatives worked through inhibition of proliferation and further induction of apoptosis.

Further work will now be focused on the possibility of inhibition of topoisomerases by using different titanocene derivatives, which would be essential for several cellular events such as DNA replication, transcription, chromosome condensation and segregations.

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Abbreviations

AO	acridin orange
CFSE	carboxyfluorescein succinimidyl ester
COSY	correlation spectroscopy
CV	crystal violet
DMSO	dimethyl sulfoxide
EI-MS	electronic impact mass spectrometry
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate (FITC)
FT-IR	Fourier transformed infrared spectroscopy
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HSQC	heteronuclear single quantum coherence
LDH	lactate dehydrogenase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS	phosphate-buffered saline
PI	propidium iodide
RPMI	Roswell Park Memorial Institute
THF	tetrahydrofuran

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jorganchem.2013.07.059.

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